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1. Your reference — C1393.00/M

2. Patent application number *(The Patent Office will fill in this part)* — 27 NOV 2001 — 0227649.1

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Medical Research Council
20 Park Crescent
London
W1B 1AL

Patents ADP number *(if you know it)* 00596007001

If the applicant is a corporate body, give the country/state of its incorporation — England and Wales

4. Title of the invention — Uses of Optical Projection Tomography Methods and Apparatus

5. Name of your agent *(if you have one)* — KEITH W NASH & CO

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

90-92 Regent Street
Cambridge
CB2 1DP

Patents ADP number *(if you know it)* — 1206001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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b) there is an inventor who is not named as an applicant, or
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Description	45 ✓
Claims(s)	
Abstract	
Drawing(s)	15 ✓

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
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Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature KEITH W NASH & CO — Date 27/11/2002

12. Name and daytime telephone number of person to contact in the United Kingdom — Dr H J Forsyth — 01223 355477

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C1393.00/M

Title: Uses of Optical Projection Tomography Methods and Apparatus

The present invention relates to uses of optical projection tomography methods and apparatus.

Methods and apparatus for optical projection tomography are described in pending International Patent Application PCT/GB02/02373 and United Kingdom Patent Applications 0214846.8, 0220157.2 and 0220156.4. The full text of the patent applications and the references cited therein are hereby incorporated by reference.

According to the present invention, there is provided a method of performing any one or more of the analyses or procedures listed hereunder comprising use of a method or apparatus of any of the aspects set out below, and as in the earlier patent applications.

There is also provided use of a method or apparatus as described in any of the aspects as set out below in any one or more of the analyses or procedures listed hereunder.

According to the present invention, the analyses and procedures of the present invention include:

Analysis of the structure of biological tissues.
Analysis of the function of biological tissues.
Analysis of the shapes of biological tissues.
Analysis of the distribution of cell types within biological tissues.
Analysis of the distribution of gene activity within biological tissues,
including the distribution of:

- RNA transcripts

- proteins

Analysis of the distribution of transgenic gene activity within biological tissues,

Analysis of the distribution of cell activities within biological tissues,

including:

- Cell cycle status including arrest
- Cell death
- Cell proliferation
- Cell migration

Analysis of the distribution of physiological states within biological tissues.

Analysis of the results of immunohistochemistry staining techniques.

Analysis of the results of in-situ hybridisation staining techniques.

Analysis of the distribution of molecular markers within biological tissues,

including any coloured or light-absorbing substances, such as:

5,5'-dibromo-4,4'-dichloro-indigo (or other halogenated indigo compounds)

formazan

or other coloured precipitates generated through the catalytic activity of enzymes including: b-galactosidase, alkaline phosphatase or other coloured precipitates formed upon catalytic conversion of staining substrates,

including: Fast Red, Vector Red

And including any light-emitting substances,

Therefore including any fluorescent substances,

such as: Alexa dyes, FITC, rhodamine,

And including any luminescent substances,

such as green fluorescent protein (GFP) or similar proteins,

And including any phosphorescent substances.

Analysis of tissues from all plant species.

Analysis of any tissue for agricultural research,

including:

basic research into all aspects of plant biology (genetics, development, physiology, pathology etc.)

analysis of tissues which have been genetically altered.

Analysis of tissues from all animal species.

including:

invertebrates

nematode worms

vertebrates

all types of fish (including teleosts, such as zebrafish, and chondrycthes including sharks)

amphibians (including the genus Xenopus and axolotls)

reptiles

birds (including chickens and quails)

all mammals (including all rodents, dogs, cats and all primates, including human)

Analysis of embryonic tissues for any purpose,

including:

research into any stem cell population

research into developmental biology

research into the causes of abnormal embryo development, including human syndromes

autopsies of human terminated pregnancies (both spontaneous and induced terminations)

Analysis of any tissues for the purpose of genomics research,

including:

the analysis of any tissues for the purpose of genomics research,

including:

the analysis of transgenic, knock-in, knock-down or knock-out organisms
the analysis or discovery of the expression (or activity) of genes including their spatial distribution, and their levels of expression
the analysis of discovery of abnormalities in the structure or morphology of tissues, as a result of interference due to wilful experimentation (such as genetic or physical modifications including a chemical or biochemical genomics approach), and/or spontaneous abnormalities (such as naturally-occurring mutations)

Analysis of any tissue for the purpose of neurobiology research,
including:
the analysis of the morphology of nerves
the analysis of the pathways and connectivity of nerves
the analysis of parts of, or whole, animal brains

Analysis of any tissue for pharmaceutical research,
including:
the analysis of pharmaceutical substances (such as drugs, molecules, proteins, antibodies),
including their spatial distribution within the tissue, and their concentrations
the analysis or discovery of abnormalities in the structure or morphology of tissues.

Analysis of tissues for medical research,
including:
research into the genetics, development, physiology, structure and function of animal tissues
analysis of diseased tissue to further our understanding of all types of diseases
including:
congenital diseases

acquired diseases

including:

infectious

neoplastic

vascular

inflammatory

traumatic

metabolic

endocrine

degenerative

drug-related

iatrogenic or

idiopathic diseases

Analysis of tissues for medical diagnosis, treatment or monitoring, including:

the diagnosis of cancer patients

including:

searching for cancerous cells and tissues within biopsies

searching for abnormal structure or morphology of tissues within biopsies

the analysis of all biopsies

including the analysis of:

lymph nodes

polyps

liver biopsies

kidney biopsies

prostate biopsies

muscle biopsies

brain tissue

the analysis of tissue removed in the process of extracting a tumour from a patient

including:

determining whether all the tumour has been removed

determining the type of tumour, and the type of cancer.

According to the present invention, samples for use in the present invention may be prepared as described in the earlier patent applications and/or employing conventional pathological and histological techniques and procedures well known to persons skilled in the art.

For example, in-situ hybridisation (particularly useful for detecting RNAs):Hammond K L, Hanson I M, Brown A G, Lettice L A, Hill R E "Mammalian and Drosophila dachsund genes are related to the Ski proto-oncongene and are expressed in eye and limb". Mech Dev. 1998 Jun;74(1-2):121-31.

Immunohistochemistry (particularly useful for detecting proteins and other molecules): Sharpe J, Ahlgren U, Perry P, Hill B, Ross A, Hecksher-Sorensen J, Baldock R, Davidson D. "Optical projection tomography as a tool for 3D microscopy and gene expression studies" Science. 2002 Apr 19;296(5567):541-5.

It will be appreciated that modification may be made to the invention without departing from the scope of the invention.

In accordance with an aspect of the present invention, there is provided a rotary stage for use in imaging a specimen from a plurality of directions, the rotary stage comprising specimen support means including a rotatable member operative to rotate a specimen to be imaged about a vertical or substantially vertical axis of rotation transverse to an optical axis along which light is emitted from the specimen, wherein

the specimen support means is disposed above a stationary imaging chamber for receiving the specimen immersed in optical imaging fluid within the chamber.

The rotary stage may be used with a separate microscope and associated hardware and software that allows three dimensional imaging of a specimen, such as a biological tissue. By having a specimen support means spaced from the microscope, the positioning of the specimen can be easily adjusted due to improved accessibility of the specimen holder. With an elongate specimen, the longest axis of the specimen is substantially parallel to gravity when held within the specimen support means. This allows the specimen to be held at one point only, again assisting with location of the specimen within the specimen support means, and avoids deflection of the specimen through gravitational effects, as such deflection can cause unwanted distortion of the specimen shape and affect the accuracy and resolution of the image obtained.

By having a stationary chamber separated from the rotational part of the stage, the chamber shape is not limited to a rotationally symmetric shape. Preferably the chamber has at least one planar face on which light impinges to image the specimen. Use of a flat planar face with no imperfections or undulations ensures that image distortion due to refraction of light is reduced. The chamber may be formed as a transparent hollow cuboid and arranged such that two opposing sides of the cuboid are substantially perpendicular to the optical axis along which light is emitted from the specimen so that a large cross-sectional area is presented to the optical axis. The selection of such a chamber with a square cross-section ensures that the amount of light refracted before passing through the specimen is substantially reduced over prior art cylindrical rotating chambers and thus image quality is improved. One wall or face of the chamber may be shaped so as to refract light in a desired way, for example to provide a magnifying effect.

The rotary stage may further comprise a pivotally mounted adjustment means, such as a lever, having a spigot extending therefrom, the spigot being arranged in use so as to engage with a specimen to alter its position relative to the axis of rotation.

The rotary stage may further comprise a prism positioned so as to receive light after the latter has illuminated the specimen, the prism acting to deflect light through 90° to enable the light to be received by a microscope with a vertical optical axis. By using a prism, the optical path to the microscope does not need to be straight, and thus modification of existing microscopes is not needed for use with a rotary stage in accordance with the present invention.

The rotatable member of the specimen support means may be carried on an adjustable platform, the position of which relative to the horizontal is variable. This allows the axis of rotation to be adjusted relative to an optical axis so that if required a 90° angle is set between the optical axis and the axis of rotation. This is particularly useful for three dimensional imaging.

The adjustable platform is preferably vertically adjustable so as to raise and lower the rotating member relative to the optical axis, so allowing a specimen to be lowered into or out of an optical path of light.

Preferably the rotatable member is formed to enable the specimen to be hung, suspended or to downwardly depend from the lower end of the rotatable member. Where a specimen is appropriately prepared with a magnetisable metal mount, attachment of the specimen to the specimen support means is then straightforward, just relying on magnetic attraction and not on a delicate fixing. This is of advantage as typically the specimens are rather small and delicate, usually with a diameter in the range 1-20mm, and securing them in a holder using a screw thread can be complicated.

In accordance with another aspect of the present invention, there is provided a method of obtaining an image of a specimen, the method comprising rotating the specimen about a vertical or substantially vertical axis of rotation transverse to an optical axis along which light is emitted from the specimen, wherein the rotating specimen is immersed in fluid within a stationary optical chamber.

According to another aspect of the invention there is provided apparatus for obtaining an image of a specimen by optical projection tomography, the apparatus comprising light-scanning means and a rotary stage for rotating the specimen to indexed positions in each of which the specimen is in use subjected to a scanning movement of incident light by the scanning means.

The incident light may be scanned in a direction perpendicular to an optical axis defined by the light passing through the apparatus.

The light scanning means may form part of a confocal scanning microscope.

According to a yet further aspect of the invention there is provided a method of obtaining an image of a specimen by optical projection tomograpy, the method comprising scanning the specimen with a light beam and detecting light emanating from the specimen to derive the image.

Preferably, the detector detects light which is unscattered.

The incident light is preferably scanned in a raster pattern, one complete scan being undertaken at each indexed position of the specimen.

According to another aspect of the invention apparatus for obtaining an image of a specimen by optical projection tomography comprises light scanning means, a rotary stage for rotating the specimen to be imaged, an optical system and light detector

means, wherein light from the scanning means scans the specimen and the optical system is operative, throughout the scanning movement of the light, to direct onto the detector means only light which is unscattered and unrefracted as a result of passing through the specimen.

The optical system is preferably a convex lens which causes convergence of incident light but directs onto the detector means all unscattered and unrefracted light, i.e. all light emerging from a specimen in a direction parallel to the central optical axis of the apparatus. A concave mirror or diffraction grating could be used instead of the convex lens.

In a preferred embodiment, the light scanning means form part of a confocal scanning microscope and the rotary stage (corresponding to the rotary stage disclosed in the applicant's co-pending International Patent Application No. PCT/GB02/02373) includes a stationary chamber within which the specimen is suspended.

The light detector means may be a localised detector positioned on the optical axis of the apparatus, so as to receive only the unscattered and unrefracted light, as previously described.

However, the light detector means may be a one-dimensional, i.e. linear, array. In this case, a central detector of the array constitutes the light detector means and the detectors on either side of the central detector constitute auxiliary detectors which detect scattered and/or refracted light. The intensities of light received by the auxiliary detectors can be used to provide information on the refractive characteristics of the specimen.

This approach can be extended to provide a two-dimensional array of detectors, with a central detector constituting the light detector means and the surrounding detectors

constituting auxiliary detectors which detect scattered and/or refracted light in the additional planes.

According to yet another aspect of the invention there is provided an optical system for use in apparatus for obtaining an image in optical projection tomography, the optical system receiving light from a specimen scanned by a light beam and being operative to direct onto a localised region only light which is unscattered and unrefracted as a result of passing through the specimen.

According to a yet further aspect of the invention there is provided a method of obtaining an image of a specimen in optical projection tomography, the method comprising moving a light beam across the specimen with a scanning motion, passing the light emanating from the specimen through an optical system, and directing the light from the optical system onto a detector which detects the light from the optical system, wherein the optical system is operative, throughout the scanning movement of the light, to direct onto the detector only light which is unscattered and unrefracted as a result of passing through the specimen.

According to another aspect of the invention there is provided apparatus for treating tissue specimens by immersion in a liquid, the apparatus comprising a first structure providing a chamber for holding the liquid, and a second structure including holding means for releasably holding the specimens, the first and second structures being relatively moveable in a direction having a vertical component between a first position in which the holding means are relatively close to the chamber and in which the second structure closes the top of the chamber to enable the specimens to be immersed in the liquid whilst the latter is protected from the environment, and a second position in which the holding means are relatively distant from the chamber to enable the specimens to be loaded onto or unloaded from the holding means.

Preferably, the first structure is stationary and the second structure is shiftable vertically with respect to the first structure.

The holding means may include magnets to enable specimens, each provided with a metal mount, to be detachably retained on the second structure by magnetic attraction.

The chamber is preferably in the form of an annular trough in which case the holding means may hold the specimens so that the latter depend from the holding means, conveniently at angularly spaced positions around a circle such that the specimens are lowered into the trough as the second structure is lowered to its first position. In this case, the second structure preferably includes a lid which acts to close the chamber in the first position and the underside of which carries the holding means. Lid closure helps to prevent evaporation of volatile treatment liquids.

The second structure may be rotatably moveable around a central vertical axis, enabling specimens to be loaded onto and unloaded from the second structure at a chosen position alongside the apparatus, either by a robotic arm or a human hand.

The apparatus may have the facility to change the liquid when in the first position, enabling the specimens to be treated by different liquids in a succession of treatment stages, whilst retaining the chamber closed. For example, the apparatus may have a pump to fill and empty the chamber with a succession of chosen liquids which, in the case of tissue specimens, may act to wash or otherwise treat the specimens prior to the specimens being imaged by means of optical projection tomography.

According to yet another aspect of the invention there is provided a method of treating tissue specimens by immersion in a liquid in a chamber, the method comprising loading the specimens onto a holder so that the specimens depend from the holder and are disposed above the liquid in the chamber, effecting relative movement between the chamber and the specimens in one direction to cause immersion of the specimens in the

liquid whilst maintaining the chamber closed and protected from the environment during immersion, effecting relative movement between the chamber and the specimens in the opposite direction to bring the specimens out of the liquid, and unloading the treated specimens from the holder.

The specimens may be treated by different liquids in a plurality of treatment stages which are preferably carried out by successive emptying and filling of the chamber with the different liquids, whilst the specimens remain in the chamber and whilst the chamber remains closed and protected from the environment.

Brief Description of the Drawings

The invention will now be described, by way of example, with reference to the accompanying drawings in which:

Figures 1 to 18 relate to an aspect of the invention in which:

Figure 1 is a perspective view of optical imaging apparatus comprising a rotary stage in accordance with the present invention together with a microscope;

Figure 2 shows a schematic illustration of how such imaging apparatus is controlled when acquiring digital images;

Figure 3 shows a front perspective view of the apparatus;

Figures 4(a) and 4(b) are schematic diagrams used to illustrate the most appropriate working configuration of the apparatus;

Figures 5(a), 5(b), 5(c) and 5(d) illustrate attachment of a specimen to the specimen support means and alignment of a region of interest;

Figures 6(a), 6(b) and 6(c) are schematic diagrams used to explain resolution of the apparatus;

Figures 7(a) shows a cross-section through a prior art specimen containing tube, with Figures 7(b) and 7(c) showing two specimen chambers as used in the present invention;

Figures 8(a) and 8(b) show a partial plan view along line VIII-VIII of Figure 1, illustrating use of a pivotally mounted lever to adjust specimen position;

Figure 9 is a perspective view of modified optical imaging apparatus according to the invention,

Figure 10 is a diagram illustrating the apparatus of Figure 9,

Figures 11a, 11b, 11c, 12a, 12b, 13a, 13b and 14 illustrate positioning and viewing of the specimen image in the apparatus of Figure 9,

Figures 15 and 16 illustrate a collimated illumination means which may be used in the apparatus of Figure 1 or Figure 9,

Figure 17 indicates a way of selecting wavelength from a light source in the optical stage of Figure 1 or Figure 9,

Figure 18 illustrates a modification of the apparatus shown in Figure 16,

Figures 19 to 30 relate to a further aspect of the invention in which:

Figure 19 is a diagram of the apparatus,

Figures 20a and 20b show how the microscope optics of the apparatus can be arranged to have low numerical aperture or high numerical aperture,

Figure 21 shows known image-forming optics,

Figures 22 and 23 show the image-forming optics of an optical system of the inventive apparatus,

Figures 24a, 24b, 24c and 24d show representative light paths for the optical system of the inventive apparatus,

Figures 25a, 25b and 25c illustrate how different degrees of refraction affect operation of the optical system,

Figure 26 illustrates how refraction is measured using a one-dimensional array of detectors,

Figures 27 to 30 illustrate, in three dimensions, the operation of the optical system,

Figures 31 to 37 relate to a further aspect of the invention in which:

Figure 31 is an isometric view of apparatus according to the invention and in an open condition,

Figure 32 is an isometric view of the apparatus in a closed condition,

Figure 33 is a fragmentary view, to an enlarged scale, of part of the apparatus in an open condition, showing specimens held by the apparatus,

Figure 34 is a cross-sectional view of the apparatus,

Figure 35 shows the apparatus in combination with a robotic arm,

Figure 36 is an enlarged view of part of Figure 35, and

Figure 37 shows the robotic arm positioning a specimen onto the rotary stage of an OPT scanner.

Detailed Description

Figure 1 shows optical imaging apparatus in the form of an OPT scanner comprising a rotary stage 10 and a long working-distance or dissecting microscope 12, separate from the rotary stage 10. The rotary stage 10 has a support 14, a pivotally mounted lever 16, an iris and optical diffuser 20, and a quartz prism 22.. The support 14, iris and diffuser 20, and prism 22 are fixed to a base 24 of the stage 10, as is a holder 25 for receiving a transparent chamber 26, or cuvette, of a generally cuboid shape. The cuvette 26 contains a fluid with suitable optical properties for imaging a specimen 28 suspended within the cuvette, an appropriate fluid being a mixture of benzyl alcohol and benzyl benzoate. This apparatus can be used for brightfield, darkfield and fluorescence imaging but is particularly appropriate where a three dimensional (3D) image of the specimen is created from a series of images taken at different angles, and for specimens too large to be imaged by confocal microscopy.

Light passes along optical axis 29, passing through the centre of the iris and diffuser 20, and through the specimen 28 and is deflected through right angles by the prism 22 to enter an objective 30 of the microscope 12. As the microscope has a large working distance, enough space is available for the prism 22 to rest beneath the microscope objective 30. Using a prism allows a vertically oriented microscope to image the

specimen. However the prism 22 can be omitted where the microscope objective is parallel to the optical axis. The iris and diffuser 20 control the amount of light passing from a light source (not shown) to reach the specimen 28 and provide even illumination.

The support 14 carries a circular boss on which is pivotally mounted, about an axis 90 (Figure 4), a tilting plate 33 upon which is slidable, upwards and downwards, a plate 32. The plate 32 carries an adjustable platform 34 cantilevered horizontally from the plate 32. The angle of the platform 34 can be altered relative to the horizontal using a tilt adjuster 36 and the vertical position of the platform 34 can be varied by means of a vertical adjuster 40. A stepper motor 42 is mounted on the platform 34, with a rotatable motor shaft 44 of the motor extending through the platform 34. A magnet 46 (a permanent magnet or an electro-magnet) is attached to the lower end of the shaft 44 and carries the specimen 28 to be imaged. The manner in which the specimen is attached to the magnet will be described later with reference to Figure 5. The stepper motor 42 rotates the shaft 44 with a step size of 0.9 degrees, providing up to 400 imaging positions of the specimen. A series of digital images of the elongate specimen 28 is taken by indexing the shaft 44 to its successive rotational positions, and thus positioning the specimen in successive rotational positions whilst the specimen is suspended within the cuvette 26, the cuvette remaining stationary.

By mounting the stepper motor 42 with its axis of rotation vertical, the rod-like specimen 28 only needs to be secured at one point, typically its uppermost end, for controlled rotation of the specimen to occur. The specimen 28 is immersed in the liquid, supported from above by the magnet 46, by using the vertical adjuster 40 to lower the platform. This vertical orientation of the specimen and the rotational axis avoids the use of O-rings or other mechanical arrangements which would be necessary to connect the dry motor to the immersed specimen, and secondly it ensures that the specimen is not deflected off its axis of rotation by gravity as the elongate specimen has its major axis parallel to the force of gravity. Avoiding distortion effects to the

specimen by having a vertically orientated specimen is particularly important for obtaining accurate 3D images, particularly for larger specimens. Use of a generally upright hollow cuboid as the imaging chamber 26 around the specimen 28 ensures that the surface area of the imaging liquid is limited, reducing evaporation of the liquid. In addition much larger specimens, typically 1-20mm in diameter, can be imaged by using such a fixed chamber without loss of digital signal quality.

In use, a digital camera 52 (Figure 2) is attached to the microscope 12 and produces a digital image of the specimen as imaged by the microscope from light that has travelled along the optical axis 29, and been transmitted through the chamber and specimen. A series of digital images are taken of the specimen from different angles and this digital information is fed into an algorithm which uses a mathematical formula to reconstruct the structure of the specimen in three dimensions. Typically the images are obtained using the control elements as set out in Figure 2. Thus a computer 50 carrying digital image acquisition software is in two-way communication with the digital camera 52 attached to the microscope 12 which receives images from a specimen of interest. The computer 50 controls filter wheels 56 attached to the microscope 12 to alter the wavelength of radiation that is detected. The computer acquisition software is shown diagrammatically in Figure 2 as software 58 to control image-capture from the digital camera, a program 54 to control the imaging software, the rotary stage and the filter wheel software, software 48 to control the filter wheels and software 64 to convert the image files into a 3D reconstruction. The computer is also in two-way communication with electronic control circuits 60 connected to the rotary stage 10 and controls the circuits 60 to adjust the orientation of the specimen as required during image capture of successive images. Once the digital images have been obtained, they are processed at 64 to produce a 3D reconstruction 66 of the specimen using mathematical processing, in a similar manner to the analysis described in US 5,680,484.

If required, the computer can control the entire imaging process, undertaking image-processing to determine the size of the specimen, its alignment, whether it is in focus

etc., and adjusting the specimen position before performing the rotational imaging. This complete automation of the imaging process is particularly desirable for large scale gene expression mapping projects in which many such devices could be run in parallel.

The circuitry 60 responsive to the computer to control the stepper motor 42 is commercially available for most popular computer systems. The circuitry 60 connects to the computer 50 and is responsive to signals from the computer 50 to control a variety of mechanical devices (stepper motors, solenoids etc.).

To create a 3D representation of the specimen, software performs the following functions: (1) determine the axis of rotation (through the symmetry which exists between each pair of images which were taken at 180 degrees to each other), (2) reorganise the stack of images into an orthogonal stack of projection images (in which image represents a single section through the specimen, viewed from all the different angles captured), (3) perform the mathematical processing on each projection image, to recreate that section through the specimen, (4) combine all the calculated section images into a 3D format. Reconstructions can be created both from transmitted light and from fluorescently-emitted light.

Now that the general apparatus and its use in data acquisition has been described, certain components of the imaging apparatus will be described in more detail.

A front view of the rotary stage 10 is shown in Figure 3. The tilt adjuster 36 varies the angle of tilt of the platform 34 about the axis 90 which is below the lower end of the shaft 44 and is approximately at the height of the specimen so that tilt adjustment does not move the specimen substantially. The axis 90 may intersect the optical axis 29. Tilt adjustment (illustrated by the double-headed arrow 92 in Figure 3) ensures that the rotational axis 94 of the stepper motor 42 is accurately perpendicular to the optical axis 29. Having adjusted the tilt of the platform 34, the position of the platform 34 relative to the base 24 is adjusted using the vertical adjuster 40 which uses a rack and pinion

arrangement to raise and lower the platform 34 in the adjusted direction of the rotational axis 94. By using the vertical adjuster 40, a specimen carried on the magnet attached to the end of the shaft 44 can be lowered a required depth into the imaging chamber for imaging and raised out of the chamber once imaging has been performed. The vertical position of the specimen during imaging an also be altered in this way if required. In the raised position of the shaft, specimens can be loaded into or out of the rotary stage.

When the apparatus is set up, it is aligned such that the optical axis of the microscope passes through the prism, and through the centre of the imaging chamber. However, at high magnification the alignment can need adjusting as the specimen becomes slightly displaced away from the centre of the field-of-view. The raising/lowering mechanism mentioned above can be adjusted to correct for this misalignment in the vertical direction.

Whilst much imaging of the specimen can be undertaken by having the rotational axis approximately perpendicular to the optical axis, 3D reconstruction of the specimen using the mathematical processing will be of very poor quality unless the angle between the optical axis and the rotational axis is exactly 90°. The tilt adjuster 36 allows the axis of rotation 94 to be tilted slightly so as to ensure the angle is exactly 90°. The tilt adjuster 36 typically relies upon a screw-thread mechanism to urge the platform 34 to one side. A calibration sample is used to adjust the angle of tilt, with the calibration sample containing a number of small particles whose trajectories can be monitored on a computer screen while the shaft rotates. If the axis of rotation is not perfectly perpendicular to the optical axis, the trajectory of the particle appears as an ellipse, see Figure 4(a) which shows the view along the optical axis as the shaft rotates about the axis 94. When the axis is correctly aligned, the particle is seen to move from side to side, with no vertical component to the motion, see Figure 4(b).

Figure 5 shows the magnetic mounting system used which relies upon magnetic attraction between a metal disc 110 attached to a specimen 112 and the cylindrical magnet 46 permanently attached to the lower end of the rotatable shaft 44 of the stepper motor 42. Each specimen has a small magnetisable metal disc glued at one end during specimen preparation. The disc is then attached to the magnet when imaging is to be undertaken and the specimen supported as a result of the magnetic attraction between the disc and the magnet. As the disc 110 and specimen 112 are relatively light, the magnet does not need to be strongly magnetised to support their weight. One advantage of the magnet system over, for example, a screw-in system, is that the small size of the disc and specimen necessitates handling with forceps or tweezers. Placing the mount or disc 110 onto a magnet is straightforward with forceps, whereas screwing it into an attachment is not. Another advantage is that the position of the specimen relative to the axis of rotation can be readily adjusted by sliding the mount 110 across the magnet surface 120. Also many specimens can be pre-prepared with a disc attached, and then quickly fitted into the device for imaging when required.

Certain liquids used in the chamber for sample imaging are toxic and corrosive to plastic, and where this is the case, the specimens are best handled using forceps. The magnetic attachment system is then of advantage as the specimens need only be held under the magnet to become securely attached. It is equally easy to remove each specimen after imaging.

To maximise the resolution of the images, a region of interest 122 in a specimen 112 must be centred on the axis of rotation 94, i.e. not move as the shaft rotates. If the region of interest, or the whole specimen, is off-centre and oscillates from side-to-side during a rotational image capture, then the magnification necessary to keep it in view will be low. This is illustrated in Figure 6(a). The two shapes 130, 132 represent the specimen 112 during rotation, at its most extreme positions to the left and right. When the specimen 112 is perfectly centred, it spins on its own axis, see Figure 6(b). This

presents a smaller width across the field-of-view, and so the magnification can be increased to provide an image with higher resolution, see Figure 6(c).

Adjustment of the specimen 112 relative to the axis of rotation 94 is simplified by the magnetic attachment. By pushing on the disc 110, the centre of the disc can be offset relative to the rotational axis 94. In Figure 5(a) the region of interest 122 within the specimen 112 is not centred on the axis of rotation but rather is displaced to the left. If the motor shaft is rotated through 180°, the region of interest 122 is now visible on the right hand side of the axis of rotation, see Figure 5(b). Because the magnet 46 allows the metal mount 110 to slide along it in any direction, without becoming unattached, a push from the side by the lever 16 (indicated by arrow 114 in Figure 5(c) is able to position the specimen so that the region of interest 122 is centred on the axis of rotation, see Figure 5(c). A further rotation of 180° shows that now the whole specimen 112 oscillates from side-to-side while the region of interest remains centred, see Figure 5(d). Adjustment of the specimen in this way is usually undertaken whilst observing images of the rotating specimen on a computer screen.

The imaging chamber 26 as shown in Figure 1 will now be described in more detail with reference to Figure 7. By having a fixed specimen chamber that does not rotate with the specimen during imaging, the chamber does not need to be cylindrical to maintain a constant optical path during rotation, as for the system described in US 5,680,484. A comparison of prior art tube 136 and the chamber used in the present invention is shown in Figure 7, Figure 7(a) showing a cross-section through the prior art cylindrical tube 136 (which is suspended horizontally), and Figure 7(b) showing the chamber 26 used in the present embodiment. The imaging chamber 26 is chosen to be generally cuboid and to be square in cross-sectional shape, and is made from quartz, glass or other suitably transparent material. Each chamber/tube contains a specimen 141 bathed in liquid 143 with suitable optical properties to allow imaging of the specimen. The flat sides 142, 142' 144, 144' of chamber 26 reduce refractive distortion of the image and allow larger specimens to be imaged. This is because the

mutually parallel walls 142, 142' of the square cross-section chamber are aligned perpendicular to the optical axis 29 and provide a greater imaging area over which non-refraction of light occurs than for the circular tube 136, which only has a very small part of its circumference at normal incidence to the light. Thus a good image can be formed across a width of more than 10mm for the chamber 26, improving the amount of signal received from the sample and reducing distortion due to refraction.

Figure 7c illustrates a modification of the sample chamber of Figure 7b. In Figure 7c, the sample chamber 26' has a square internal cross-section but one wall 140 is shaped to provide a plano-convex lens to refract light leaving the chamber. The shaping causes a desired refraction, in the case of Figure 7c a magnifying effect.

The lever 16 shown in Figure 1 is now described in more detail with reference to Figure 8, which shows a plan view along line VIII-VIII of Figure 1. Figure 8(a) shows the lever 16 in its usual position, pushed away from the magnet 46 and metal specimen mount 110. If the specimen is displaced too far to one side (as illustrated) the lever 16 can be moved about pivot 164 so that spigot 166 engages with metal mount 110 to push the specimen into the correct position (Figure 8b). This is done while the specimen position is monitored on the computer screen. Since the stepper motor can be carefully controlled through manual switches, the specimen trajectory during rotation can be observed and the motor stopped when the specimen is maximally to one side. The specimen is then centred using the lever 16, and the process repeated until alignment of the specimen relative to the optical axis is complete. The lever 16 is organised so as to produce a "geared-down" movement to the specimen, which makes it easier to control the adjustment.

The pivot 164 is attached to the main motor stage. It is fixed to the stage by a support which ensures the spigot 166 is at the correct height to contact the metal mount, just below the magnet. This way, the spigot 166 remains at the correct height irrespective of the height chosen to image the specimen.

The apparatus described herein is suitable for 3D microscopy and also rotational microscopy for any purpose, on biological specimens and specimens from other fields such as material science.

When undertaking 3D microscopy, the refractive index should be uniform throughout the specimen. For biological tissue this is easily achieved by bathing the specimen in a clearing solvent. A specimen can be glued directly onto the metal mount, or embedded in a block of transparent matrix such as agarose, which is itself adhered to the mount. The clearing solvent then permeates the blocks as well as the specimen. BABB (a mixture of benzyl alcohol and benzyl benzoate) is suitable as a solvent.

For a specimen whose refractive index cannot be made uniform, or which is not transparent, the technique is still of use. The 3D surface shape of objects whose cross-sections are all convex (even if the whole 3D shape is not convex) can accurately be recreated from its rotating silhouette.

There are some applications where the raw data of the apparatus is useful. The series of images can be converted into a movie of the rotating object (i.e. the specimen). It is much easier to grasp the shape of a 3D object when it is viewed rotating than from a few static 2D images (many 3D reconstruction projects present their results as movies of a model rotating).

The apparatus is also suitable for undertaking 3D mapping of gene expression patterns (RNA and/or protein distribution) in biological tissue, whilst allowing the specimen to be used for other analysis after imaging. Specimen imaging using the apparatus is relatively quick, taking around 20 minutes. In contrast preparing, embedding, sectioning, mounting, staining and digitising real histological sections takes days and produces hundreds of digital 2D sections, but no guaranteed way to align them with each other to recreate the original 3D shape. The histological sections tend to stretch

significantly, such that even if all the sections can be fitted onto each other to create a 3D shape, the final result will not accurately reflect the shape of the original specimen. However the results obtained using the apparatus are very similar to the real physical shape of the specimen, the only difference from physical sections being reduced resolution. As the data generated by the apparatus is genuinely 3D it can be virtually resectioned in any orientation, or rendered in 3D.

A modified construction of rotary stage is illustrated in Figure 9 where parts corresponding to those of Figure 1 bear the same reference numerals. In the rotary stage of Figure 9, three-dimensional adjustment of the position of the stepper motor 42 is achieved by the use of three secondary stepper motors 150, 152, 154. No tilt adjuster for the motor 42 is present. Instead, the prism is capable of being manually adjusted by controlled tilting about a transverse horizontal axis 23. The important stepper motors are the motors 150 and 154. The motor 152 can be replaced by a manual vertical adjuster 40.

The secondary stepper motors 150, 152, 154 allow sub-micron accuracy adjustment of the 3D position of the primary stepper motor 42, along the orientations labelled as x, y and z. These stepper motors 150, 152, 154 are controlled by the same computer which controls the primary motor 42. This is illustrated in Figure 10 where the computer 50 drives the four motors through motor driving circuits 156. For the purposes of this document, the z-axis is considered parallel to the optical axis 29. Movements along this axis effectively alter the focus of the system. Movements along the other two axes alter which part of the specimen coincides with the centre of the optical axis 29.

The computer-controlled translation by the three secondary motors 150, 152, 154 has the following advantages:

1) It allows the region of interest (ROI) of the specimen to be maintained centrally within the field-of-view of the microscope. This is achieved in two ways:

(a) The ROI is maintained within the depth-of-focus of the microscope.

(b) It limits the "side-to-side" oscillatory movements of the ROI along the x-axis.

These two advantages allow much higher resolution imaging as compared to a system which has no such mechanism.

2) It is more accurate than the lever and spigot system of Figures 1 and 8.

3) It can be controlled completely by the computer (unlike the lever and spigot system), so the ROI can be easily defined "on-screen" within the software.

4) It allows the computer to calculate precise 3D coordinates for the ROI.

5) It allows different scans within the same specimen to be related to each other in 3D space.

6) This allows the computer to build-up a high resolution scan of a large specimen from multiple automatic scans of smaller regions at higher magnification (known as "tiling" or "patching").

Computer controlled x and z movements to maintain the ROI within the field-of-view are calculated as follows:

First, the software needs to calculate the positions of:

(a) The axis of rotation of the primary stepper motor 42 relative to the field-of-view.

(b) The ROI relative to the axis of rotation of the primary stepper.

These two positions can be calculated from one operation. The magnification is set low enough such that during a full rotation the ROI stays within the field-of-view of the camera. The system is previously calibrated such that it is known how many pulses to the x-stepper motor correspond to a given displacement as measured in pixels on the computer screen. This relationship is determined for each magnification. The computer then presents the user with four images of the specimen, rotated to 0, 90, 180 and 270 degrees (as seen in Figures 11a to 11c). In Figure 11a, each outer rectangle represents the imaging window on the computer screen and the spot represents the region of interest 122 of the specimen.

Figure 11b shows views along the optical axis (as seen on the computer screen) for low magnification, and Figure 11c shows plan views along the axis of rotation 94. The user then uses the computer mouse (or equivalent) to indicate where the ROI is in each image.

Figure 12 shows how the positioning system can move the stepper motor 42 in both the x and z dimensions, and can therefore compensate for the ROI being off-centre. The x and z movements of the motor 42 are controlled by the computer to ensure that the ROI 122 remains in a fixed position, rotating around an effective axis of rotation.

In Figures 11a, 11b and 11c:

$\chi 1$ = the x-position of the ROI at 0 degrees, converted to stepper motor units.
$\chi 2$ = the x-position of the ROI at 180 degrees, converted to stepper motor units.
χw = the width of the imaging window, converted to stepper motor units.

The average $\chi 1$ and $\chi 2$ provides the position (χs) of the axis of rotation of the stepper motor relative to the imaging window (χs). The average of $Z1$ and $Z2$ provides a second estimate of this position ($\chi s = (\chi 1 + \chi 2 + Z1 + Z2)/4$). The x-displacement which would be necessary to centre the axis of rotation of the stepper motor within the imaging window is:

$$\text{X-displacement } (\chi d) = \chi w/2 - (\chi 1 + \chi 2 + Z1 + Z2)/4$$

This is illustrated in Figures 13a and 13b.

In Figures 13a and 13b, the microscope views the specimen from the bottom of the diagram. The edges of the field-of-view therefore appear as two substantially parallel lines, which indicate the limits of what can be seen. The optical axis, which is the centre of this field-of-view, is shown as a vertical dashed line in Figure 13a.

Figure 13b shows the specimen at a rotational position of 0 degrees (α=0). From the measurements described on the previous page ($\chi 1$, $\chi 2$, $Z1$, $Z2$) the x and z distances of the ROI from the axis of rotation of the primary stepper motor can easily be calculated. $\chi \alpha o$ is the x-distance when the rotational position (angle α) is zero ($\chi \alpha o = (\chi 1 - \chi 2)/2$). Similarly, $Z\alpha o$ can be calculated from the two measurements taken at α=90 degree and α=270 degrees, ($Z\alpha o = (Z1 - Z2)/2$). The position of the ROI can then be converted from cartesian coordinates to polar coordinates where D is the distance of the ROI from the stepper motor axis, and θ is the angle of that line to the optical axis (or a line parallel to it), when $\alpha = 0$ degrees.

D = square root of $(\chi \alpha o^2 + Z\alpha o^2)$ $\theta = \tan^{-1} (\chi \alpha o / Z\alpha o)$

Now, for any rotational position of the primary stepper motor (α) the ROI can be positioned on the optical axis by movements of the secondary x z stepper motors, in which the total displacements (Xt and Zt) are calculated by:

$$\chi t = \chi d + D.\sin(\alpha + \theta), \quad \text{and} \quad Zt = D.\cos(\alpha + \theta).$$

The 3-D shape of the region sampled from one OPT reconstruction is substantially a cylinder with a circular cross-section, whose axis of rotational symmetry is the effective axis of rotation used during imaging, and whose diameter and length are described by the width and height of the field-of-view. Since we can alternate between cartesian and polar coordinates to describe positions within the specimen, and can relate the sizes of pixels to real distances within the specimen, we can easily calculate the position and shape of the sampled cylinder relative to any other scans made of the same specimen.

In 2-D imaging, a high-resolution image is often constructed by taking many high magnification images of small regions of the object, and then joining the smaller images together. This is often known as "tiling" or "patching". The computer-controlled XYZ stage allows the same approach to be applied to 3-D OPT imaging.

As described above, the sampled region from an OPT scan is a cylinder with circular cross-section. Figure 14 illustrates, in plan view looking down along the axis 94, how a specimen 160 can be imaged in one scan 162 at low-resolution, or alternatively could be imaged by positioning seven high-resolution scans 170 such that every position within the specimen is contained within at least one sampled region. Since the individual sample regions have a circular cross-section, one efficient arrangement for covering a large region is to arrange the scans in a hexagonal pattern, with slight overlaps between adjacent scans. Different positions along the y-axis of the specimen can also be sampled using the y-axis stepper motor.

This tiling process can be completely controlled and performed by the computer.

For all specimens which are to be imaged in their entirety with one scan, calculating the position of the optimal sampled region can be done automatically without the need for the user to identify the ROI as previously described. Simple image-processing can find the outline or the centre of the specimen within test images during the alignment process, as follows:

1) Set magnification to low (can be done automatically using a computer-controlled microscope).

2) Take four images at 0, 90, 180 and 270 degrees rotation.

3) Calculate a histogram of each image to determine a suitable threshold level to distinguish the specimen from the background.

4) Calculate the position of the centre-of-mass of the specimen in each image.

5) Use these positions as the ROI measurements as previously described.

6) Apply the new displacements during any subsequent rotations.

7) Increase magnification.

8) Take four rotated images and determine whether magnification is too high (i.e. if edges of specimen are outside of the field-of-view).

9) If specimen still within field-of-view go back to step 4.

10) If edges of specimen are outside field-of-view reduce magnification to previous value.

11) Scan specimen.

A collimated illumination means, which may be used in the rotary stage of Figure 1 or Figure 9, is illustrated in Figures 15 and 16.

A laser or other light source 172 is used in conjunction with a focussing means (either refractive lenses 174 or reflective mirrors) to generate a beam of light 176 in which all light rays are substantially parallel to the optical axis. Figure 15 illustrates this device in relation to the remainder of the rotary stage which, in this example, has two stepper motors 150, 154 for computer-controlled adjustment in the x and z directions rexpectively. Vertical adjustment is effected manually by vertical adjuster 40. The lens 22 is capable of tilt adjustment about axis 23.

As a result of experiments it is clear that illuminating light which enters the specimen non-parallel to the optical axis introduces noise into the results. A collimated light source, where, all illuminating light rays are parallel to the optical axis, reduces this problem and therefore increases the quality of imaging.

Referring to Figure 17, a wavelength filter 178 is placed at some position between the light source 180 and the specimen 28. This may either consist of a series of different filters, each permitting the transmission of different range of wavelengths, which may be manually or automatically positioned in the lightpath. Or it may be an electronically-tunable filter.

Alternatively, two electronically-tunable liquid crystal filters may be used for fluorescent imaging to restrict the wavelengths of both the illumination light and the detected light, this possibility being illustrated by the second electrically-controlled filter 182 placed in front of a 2D array of light detectors 184.

A given chemical will absorb different wavelengths with varying degrees of efficiency. These differences can be represented as a spectrum (which describes the absorption for a large range of wavelengths). Most specimens consist of varying spatial distributions of different chemicals, and consequently different specimens are optimally imaged

using different wavelengths (or combinations of wavelengths). The described filter system allows the user to alter which wavelengths are used to image a given specimen.

Similarly, fluorescent chemicals possess one spectrum which describes the efficiency of different wavelengths to excite them, and a second spectrum which describes the abundance of different wavelengths emitted on fluorescence. The use of two electronically-controlled filters produces (at least) a 2-D parameter space for the possible combinations of excitation and emission. Such a system allows the exploration of optimal combinations to distinguish between different chemicals. This allows the 3-D histology of bio-medical samples to be imaged without the need for specific stains.

It will be appreciated that a rotary stage according to the invention need not include a prism 22, and nor need the rotary stage be used with a standard vertical microscope. Figure 18 illustrates a modification of the arrangement of Figure 15. In Figure 18 (where parts corresponding to those of Figure 15 bear the same reference numerals), the light emanating from the chamber 26 enters microscope optics and a digital camera, giving a short working distance between the microscope objective 30 and the specimen.

The specimen may be positioned by the use of a translation stage carried by the shaft 44. The translation stage has manual or computer-controlled adjustment in the x and z directions.

Referring to Figure 19, the apparatus comprises a light source 201 (in the form of a laser) which supplies light to a two-dimensional light scanning means 202, the scanning mechanism of which has a dual mirror system. Light with a scanning motion is fed through image-forming optics 203. A dichroic mirror 204 interposed between the light source 201 and the scanning means 202 directs returned light to a high speed light detector 205. The components 201 to 205 may be provided by a confocal light-scanning microscope.

Light from the optics 203 passes through a specimen 206 which is rotated within, and supported by, a rotary stage 207 which in structure corresponds to the rotary stage disclosed in the applicant's co-pending International Patent Application No. PCT/GB02/02373. The rotary stage 207 rotates the specimen 206 to successive indexed positions at each of which one complete scan of the excitation light is undertaken. After passing through the specimen 206, the light is processed by an optical system 208 which directs the light to a one-dimensional or two-dimensional array of high speed light detectors 209.

In fluorescence mode, light from the specimen 206 is returned through the optics 203 and the scanning means 202 and thence, via the mirror 204, to the high speed light detector 205. In this method of fluorescence imaging, the excitation light enters one side of the specimen and leaves the specimen from the same side thereof before being detected. It is in the transmission mode, to be described, that the components shown to the right of the stage 207 in Figure 19 are used.

The microscope optics 203 may have a high numerical aperture (Figure 20a) or may be adapted to have a low numerical aperture (Figure 20b) which is useful for some specimens to be imaged.

Figure 21 illustrates a known image-forming system. The light from any point on the focal plane 212 (within the specimen) is collected and refracted by a lens 213 towards a single point in the image plane 214. There exists a symmetry such that any point on the image plane 214 maps to a point in the focal plane 212 and *vice versa*.

By contrast, the need for an *image-forming* optical arrangement is removed in the "non-focal" optics of Figures 22 and 23 which displays no such symmetry. The non-focal optical system 208 is represented by a convex lens 215. The light from a single point on the focal plane 212 is not focussed onto a single light detector. It is diverged such that only the light which continues to travel straight through the specimen 206

along the optical axis of the apparatus (i.e. the light 216 which is not scattered) reaches the single light detector 209a positioned on the optical axis. The purpose of the lens 215 in this case is very different from Figure 21. It can only function in a light-scanning situation. The narrow light beam is scanned (e.g. in a raster pattern) across the specimen through a multitude of different positions (five of which are illustrated as the black arrows in Figure 23). The purpose of the non-focal optical system 208 (i.e. the lens 215) is to direct only unscattered and unrefracted light onto the single light detector 209a, irrespective of the scanning position of the light beam. In specimens which cause significant scattering of light the system allows a higher signal-to-noise ratio to be obtained by limiting detection of scattering light. Alternatively, the specimens with low scattering but a non-uniform distribution of refractive index the system allows this non-uniform distribution to be calculated by measuring the degree of refraction experienced by each projection.

Figures 24a to 24d illustrate some representative light paths for rays (derived from a narrow laser beam) emitted from the specimen 206 while passing through the non-focal optical system.

In Figure 24a rays scattered from a point in the centre of the specimen 206 are diverged away from the light detector 209a. The proportion of scattered rays which are detected can be adjusted by changing the effective size of the detector. An adjustable iris allows this control (which is very similar to the pin-hole in a scanning confocal microscope). Alternatively, the position of the lens can be adjusted to cause more or less divergence of the scattered rays. In optical image-forming systems, an airy disc is the interference pattern produced by the light emitted from a single point within the specimen. Optical systems which produce larger airy discs have lower resolving power, as airy discs from neighbouring points within the specimen will overlap. The concept of the airy disc is not strictly relevant to a project-measuring system like this, however a similar concept does exist. In the case of the non-focal optics described here, light from each projection creates a very broad distribution of intensities (at the position of the detector)

similar to a broad airy disc, which might suggest low resolving power. However, as only a single projection is measured at any one time even very broad distributions cannot interfere with each other.

In Figure 24b rays scattered from other points along the same line sampled in Figure 24a, are also diverged away from the light detector 209a.

In Figure 24c unscattered light from a different scanned position (black arrow) is emitted from the specimen 206 substantially parallel to the optical axis, and is therefore refracted towards the light detector 209a. As in Figures 24a and 24b, scattered light is directed away from the detector 209a.

In Figure 24d unscattered rays from any scanned position are directed onto the light detector 209a. The arrows represent successive positions of the laser beam as it is scanned across the specimen 206 in a direction perpendicular to the optical axis.

All experiments done so far with optical projection tomography have had to assume that although some of the light is scattered, the refractive index of the specimen is uniform. Recent experiments have demonstrated that a number of important specimens (including medical imaging of biopsies) display non-uniform refractive indexes. This means that the current algorithms are not accurately imaging the specimen – distortions and artefacts are introduced. The apparatus described reduces this problem by measuring information not previously available – i.e. the angle at which a light beam exits from the specimen.

In the use of the present apparatus a clearing agent (such as BABB) is used such that the majority of the light is not scattered. It is however subject to a different form of disruption – refraction. In Figure 25, scattered light is indicated by broken lines, while the main path of light is shown in solid lens. In the first example of Figure 25a this path is not bent as it passes through the specimen 206 (it is only refracted on passing

through the lens). The main path does pass through a region of the specimen with a higher refractive index than the rest (grey disc), however both the interfaces it encounters between regions of differing refractive index are perpendicular to the light path, so no refraction occurs.

In the second case of Figure 25b, the illumination beam is slightly higher and therefore the interfaces it encounters between the grey region and the white region of the specimen (different refractive indexes) are slightly displaced from perpendicular. This causes two slight refractions of the main path such that when the light emerges from the specimen it is no longer parallel to the optical axis and is directed slightly to the side of the original central light detector 209a. If auxiliary light detectors 209b are positioned on either side of the central detector 209a, these can measure the degree of refraction. Any projection will give a certain distribution of intensities along the array of light detectors, with the strongest values closer to the centre of the distribution. The system need only determine where the centre of this distribution is (usually the strongest intensity) to measure the angle at which the main light path emerged from the specimen. In the last case of Figure 25c, a different scanned position has caused greater refraction of the beam, which is reflected in a further shift along the array of detectors.

In Figure 26, an oblong region of the specimen 206 has a higher refractive index (grey shape) than the rest. Rays passing around the specimen are not refracted and so are directed to the central light detector 209a. Rays passing through the middle of the specimen (middle two rays in Figure 26) are refracted twice. The two interfaces which the light passes through (white-to-grey and then grey-to-white) are parallel with each other, and the light rays therefore exit the specimen at the same angle that they entered it. These rays are also directed onto the central detector 209a, as if they had not been refracted. Rays passing through other parts of the grey region are also refracted twice but not passing through parallel interfaces, so these rays are detected by the adjacent light detectors 209b.

The fact that some rays will be refracted and still exit the specimen 206 in the same orientation (although a different position) is not a problem. The example of Figure 26 shows only one of the many sets of projections taken through this section. Full imaging involves capturing such a data set for hundreds of orientations through the section, and the combination of all this data allows a full reconstruction of the distribution.

Figures 27 to 30 show three-dimensional views of the apparatus. In Figure 27, all un-refracted (and unscattered) rays through a two-dimensional section of the specimen are focused onto the central light detector of the array. The specimen 206 is rotated about a vertical axis between indexed positions in each of which a complete scan is undertaken.

Figure 28 shows the path of scattered or refracted light onto auxiliary light detectors.

Figure 29 illustrates that the lens (or optical system) allows the one-dimensional array of detectors 209 to capture data from a full two-dimensional raster-scan of the specimen. A row of scanned positions is always directed down or up to the row of detectors, irrespective of the vertical height of the scan.

A two-dimensional array of light detectors 209 may be used instead of a one-dimensional array, as shown in Figure 30. This would be able to measure light which is scattered above or below the plane occupied by the light rays shown in Figure 30. The data derived from the detector array 209 optics is interpreted by an algorithm.

Many different algorithmic approaches already exist for performing back-projection calculations. One approach is to use a standard linear filtered back-projection algorithm (as in US Patent 5680484). Other approaches include iterative, maximum entropy and algebraic reconstruction technique. (R. Gordon et al., "Three-Dimensional Reconstruction form Projections: A Review of Algorithms".

In Figure 31, the apparatus comprises a fixed structure 301 having a cylindrical outer casing 302 in the top of which is positioned an annular trough 303 which is open at the top. Mounted in the fixed structure is a moveable structure 304 having a central hub or spindle 305 on the top of which is mounted a disc-like lid 306 the underside of which carries a number of angularly spaced and downwardly depending cylindrical magnets 307. The hub or spindle 305 is rotatably mounted in the fixed structure for rotation of the moveable structure about a central vertical axis indicated at 308 in Figure 34. Also, the hub or spindle 305 is capable of vertical translational movement with respect to the fixed structure 301 along the vertical axis 308.

Within the casing 302 are located motors and gearing for driving the structure 304 both in rotation and translational movement, as indicated at 309 in Figure 34. The trough 303 is capable of being filled with liquids, and the structure 301 includes containers for holding these liquids and pumps for filling and emptying the trough, as indicated at 310 in Figure 34.

The magnets 307 are used to hold, in a detachable manner, tissue specimens 312 each of which has been prepared with a metal mount 313 at one end of the specimen. This allows each metal 313 mount to depend from one of the magnets 307, with the specimen 312 depending downwardly from the mount 313. When the apparatus is in the open condition (Figure 31), the magnets 307 are raised clear of the top of the trough 303 so that the specimens 312 can be attached to or removed from the magnets 307. When the apparatus is in the closed position (Figure 32), the lid 306 engages the top of the casing 302 and the specimens 312 are immersed in a liquid 314 in the trough 303.

The robotic arm 315 shown in Figures 36 and 37 is used to load untreated specimens into the apparatus and also to transfer treated specimens from the apparatus to the rotary stage 316 of an OPT scanner where the specimens are imaged.

In use, the robotic arm 315 is used to load specimens into the apparatus, each specimen being attached to the lower end of a corresponding magnet 307 by virtue of the magnetic attraction between the magnet 307 and the metal mount 313 at one end of the specimen 312, the moveable structure 304 being in the open position and being indexed in a rotational sense as the specimens are loaded. When loaded with specimens, the moveable structure is moved to its lowered or closed position, thereby immersing the specimens 312 in the liquid 314 which has been pumped into the trough 303. In this closed position, the lid 306 engages the upper rim of the outer casing 302 so that the liquid 314 is closed to the air, thereby allowing the use of a volatile liquid, unlike known apparatus which uses conveyor belts for transferring specimens through a liquid.

If it is required to treat the specimens by a succession of liquids, the first liquid is drained from the trough 303 and a second liquid pumped thereinto, without the need for the trough 303 to be opened to the air. Moreover, the attachment of the metal mounts 313 to the magnets 307 retains the specimens 312 in their hanging positions so that the specimens do not engage the bottom of the trough 303, which could damage them.

Any number of treatment stages can be carried out in this manner, the liquid being changed without the need to move the specimens and employing only a small volume of each treatment liquid.

After treatment of the specimens 312, the moveable structure 304 is raised to its upper position and the treated specimens 312 are then transferred to the rotary stage 316 by means of the robotic arm 315, the structure 304 being rotationally indexed to enable the robotic arm 315 to unload each specimen 312 in turn and to transfer the treated specimen 305 to the rotary stage 316.

Examples of liquids for treating the specimens are fixatives (such as paraformaldehyde or formalin), alcohols (in particular methanol and ethanol) and organic solvents for clearing the specimens (in particular benzyl alcohol and benzyl benzoate).

The apparatus and methods can be used in various analyses and procedures, as set out below:

Analysis of the structure of biological tissues.
Analysis of the function of biological tissues.
Analysis of the shapes of biological tissues.
Analysis of the distribution of cell types within biological tissues.
Analysis of the distribution of gene activity within biological tissues,
including the distribution of:

- RNA transcripts
- proteins

Analysis of the distribution of transgenic gene activity within biological tissues,
Analysis of the distribution of cell activities within biological tissues,
including:

- Cell cycle status including arrest
- Cell death
- Cell proliferation
- Cell migration

Analysis of the distribution of physiological states within biological tissues.
Analysis of the results of immunohistochemistry staining techniques.
Analysis of the results of in-situ hybridisation staining techniques.
Analysis of the distribution of molecular markers within biological tissues,
including any coloured or light-absorbing substances,
such as:

5,5'-dibromo-4,4'-dichloro-indigo (or other halogenated indigo compounds)
formazan
or other coloured precipitates generated through the catalytic activity of enzymes including: b-galactosidase, alkaline phosphatase or other coloured precipitates formed upon catalytic conversion of staining substrates,
including: Fast Red, Vector Red

And including any light-emitting substances,
Therefore including any fluorescent substances,
such as: Alexa dyes, FITC, rhodamine,
And including any luminescent substances,
such as green fluorescent protein (GFP) or similar proteins,
And including any phosphorescent substances.

Analysis of tissues from all plant species.
Analysis of any tissue for agricultural research,
including:

basic research into all aspects of plant biology (genetics, development, physiology, pathology etc.)
analysis of tissues which have been genetically altered.

Analysis of tissues from all animal species,
including:
invertebrates
nematode worms
vertebrates
all types of fish
(including teleosts, such as zebrafish, and chondrycthes including sharks)
amphibians (including the genus Xenopus and axolotls)
reptiles
birds (including chickens and quails)
all mammals (including all rodents, dogs, cats and all primates, including human)

Analysis of embryonic tissues for any purpose,
including:

research into any stem cell population

Analysis of tissues for medical research,

including:

research into the genetics, development, physiology, structure and function of animal tissues

analysis of diseased tissue to further our understanding of all types of diseases

including:

congenital diseases

acquired diseases

including:

infectious

neoplastic

vascular

inflammatory

traumatic

metabolic

endocrine

degenerative

drug-related

iatrogenic or

idiopathic diseases

Analysis of tissues for medical diagnosis, treatment or monitoring,

including:

the diagnosis of cancer patients

including:

searching for cancerous cells and tissues within biopsies

searching for abnormal structure or morphology of tissues within biopsies

the analysis of all biopsies

including the analysis of:

lymph nodes

polyps

liver biopsies

kidney biopsies

prostate biopsies

muscle biopsies

brain tissue

the analysis of tissue removed in the process of extracting a tumour from a patient

including:

determining whether all the tumour has been removed

determining the type of tumour, and the type of cancer.

It will be appreciated that modification may be made to the invention without departing from the scope of the invention.

Fig.1.

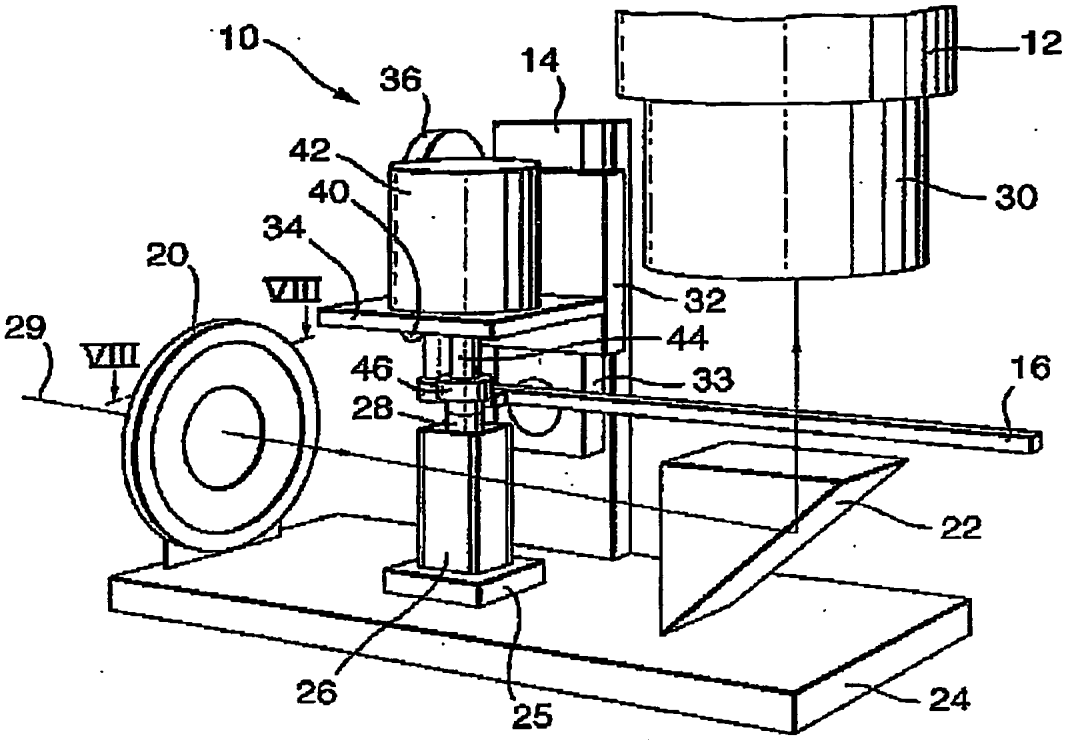


Fig.2.

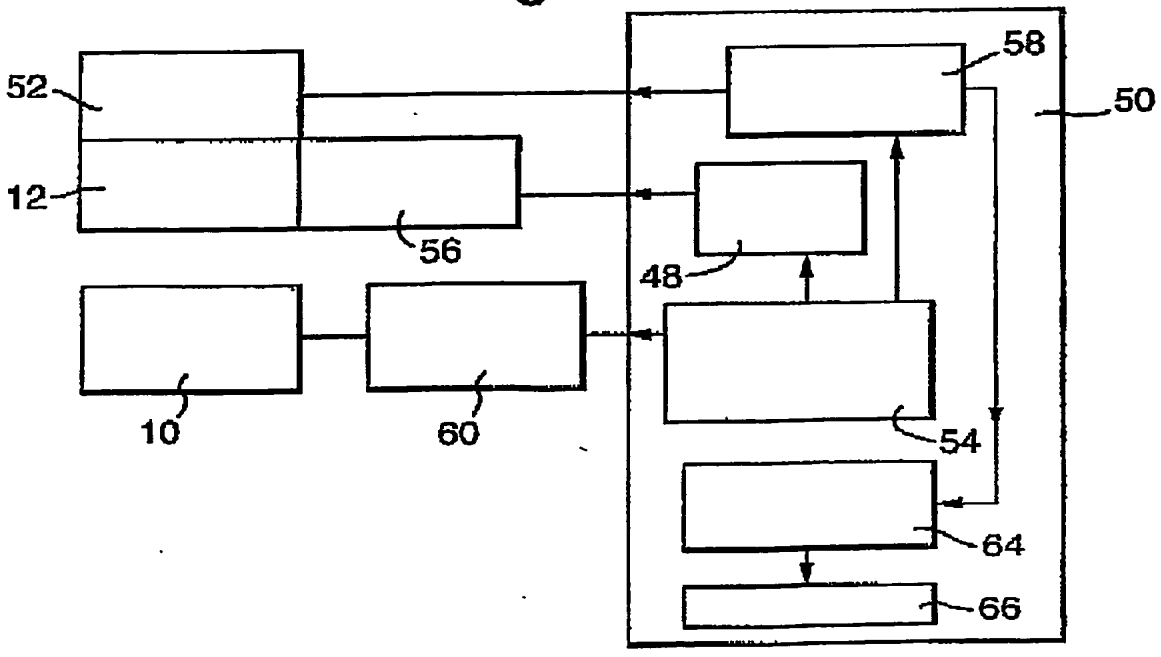


Fig.3.

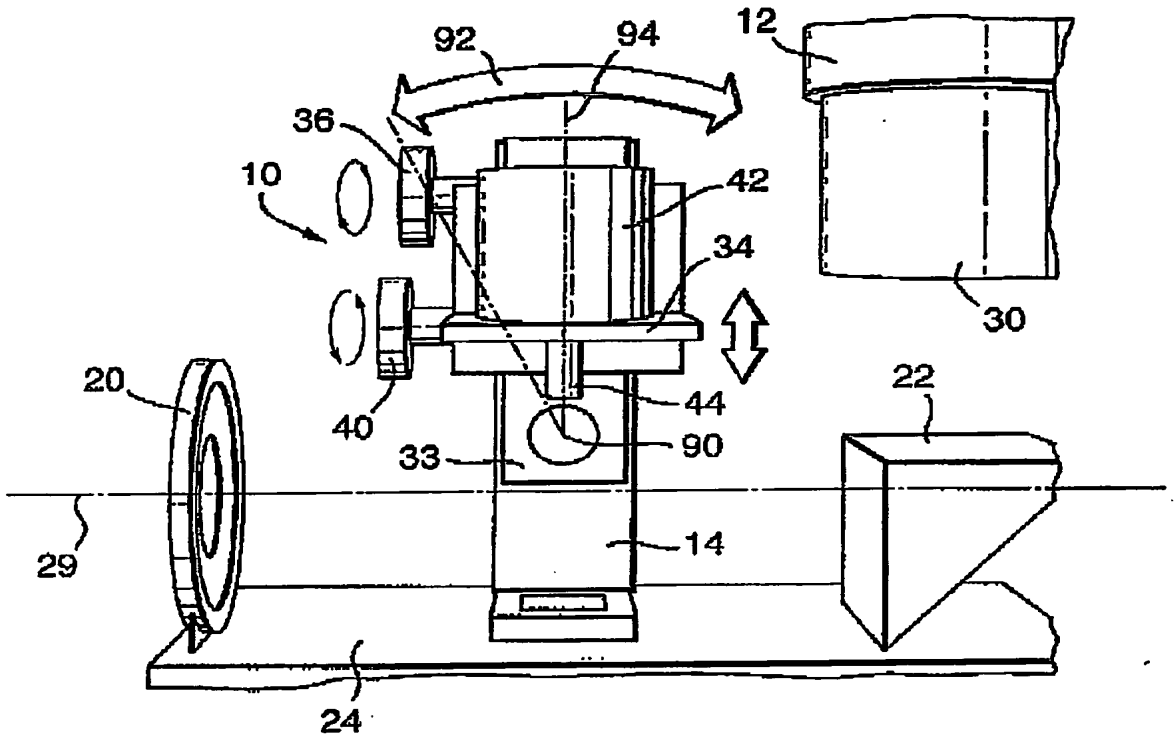


Fig.4(a)

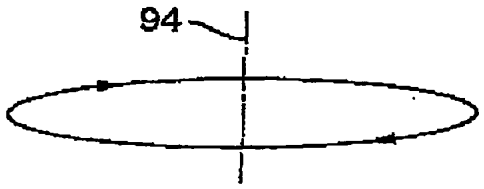


Fig.4(b)

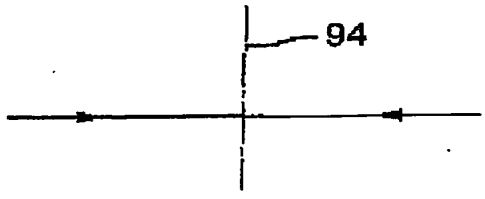


Fig.5(a)

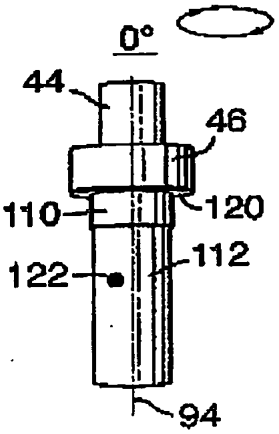


Fig.5(b)

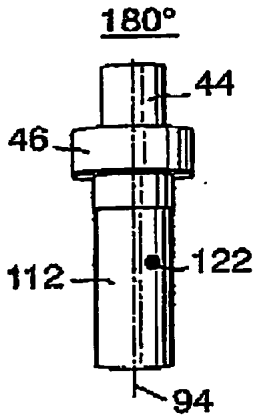


Fig.5(c)

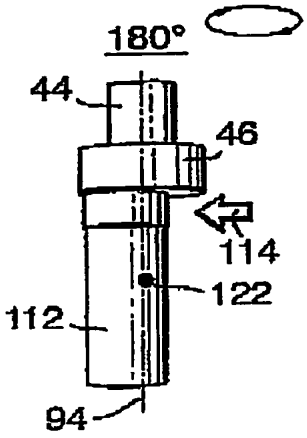


Fig.5(d)

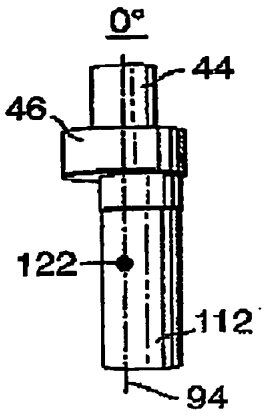


Fig.6(a)

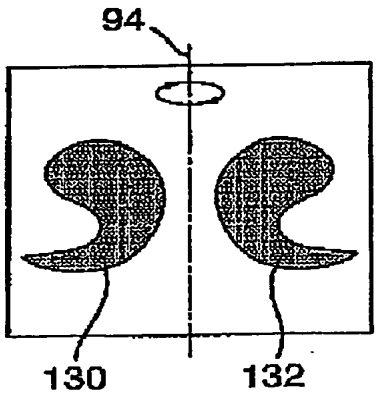


Fig.6(b)

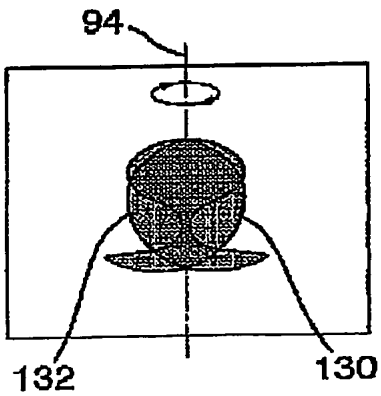


Fig.6(c)

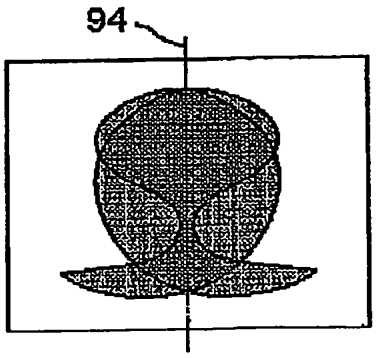


Fig.7(a)

Prior Art

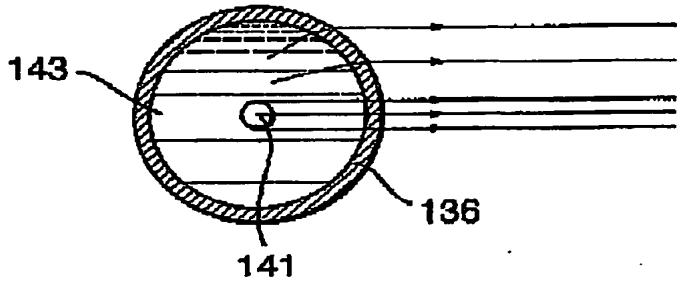


Fig.7(b)

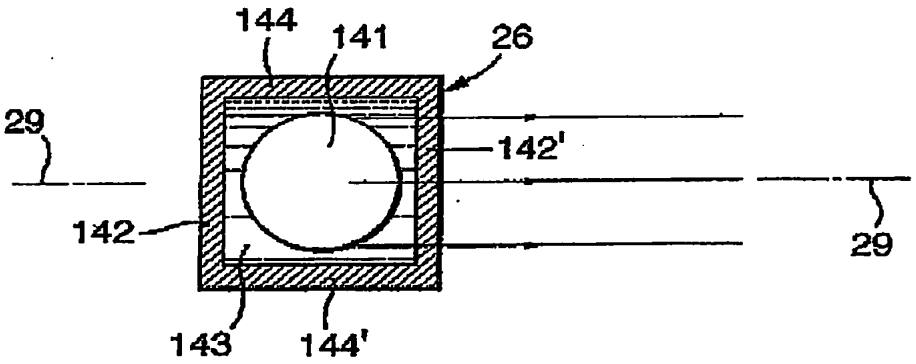


Fig.7(c)

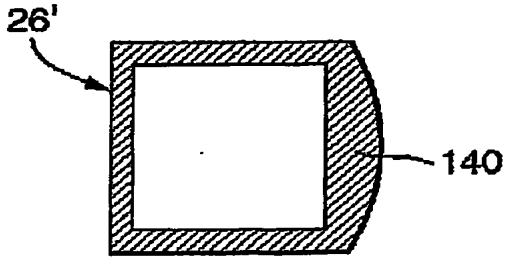


Fig.8(a)

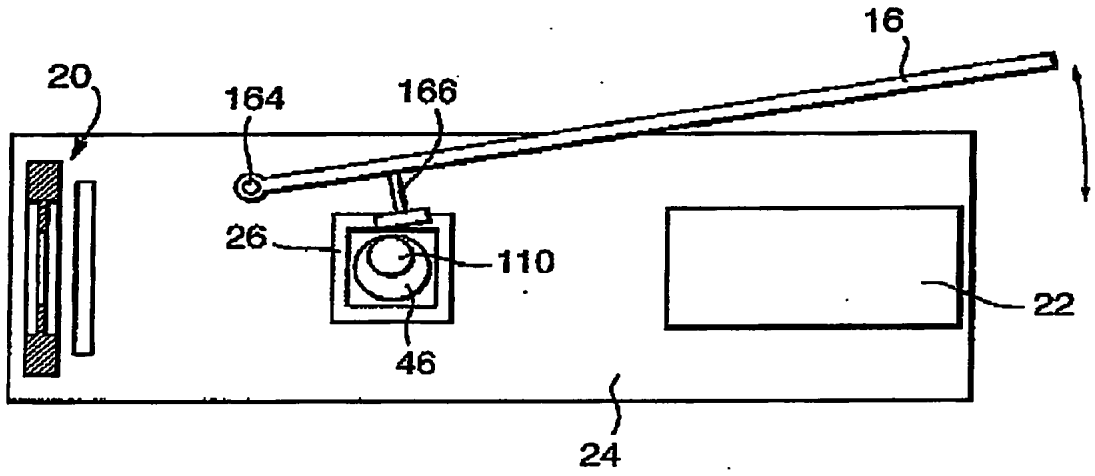


Fig.8(b)

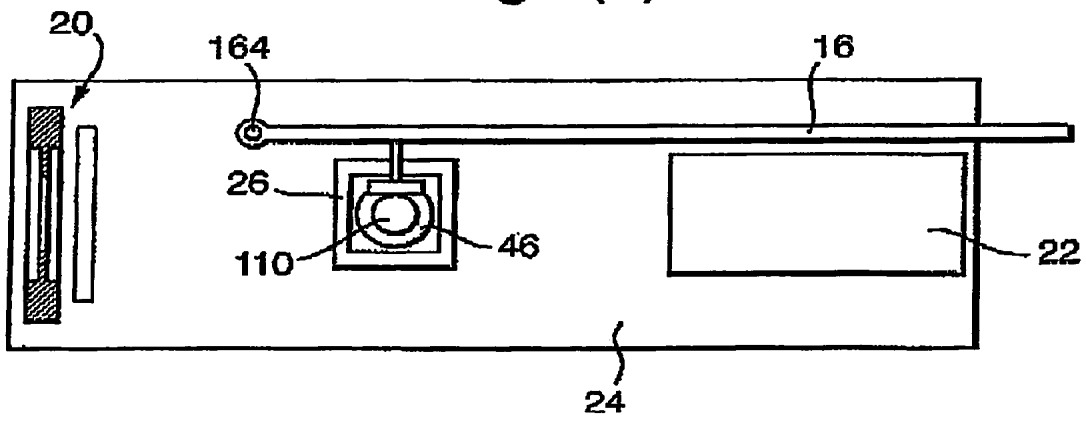


Fig.9.

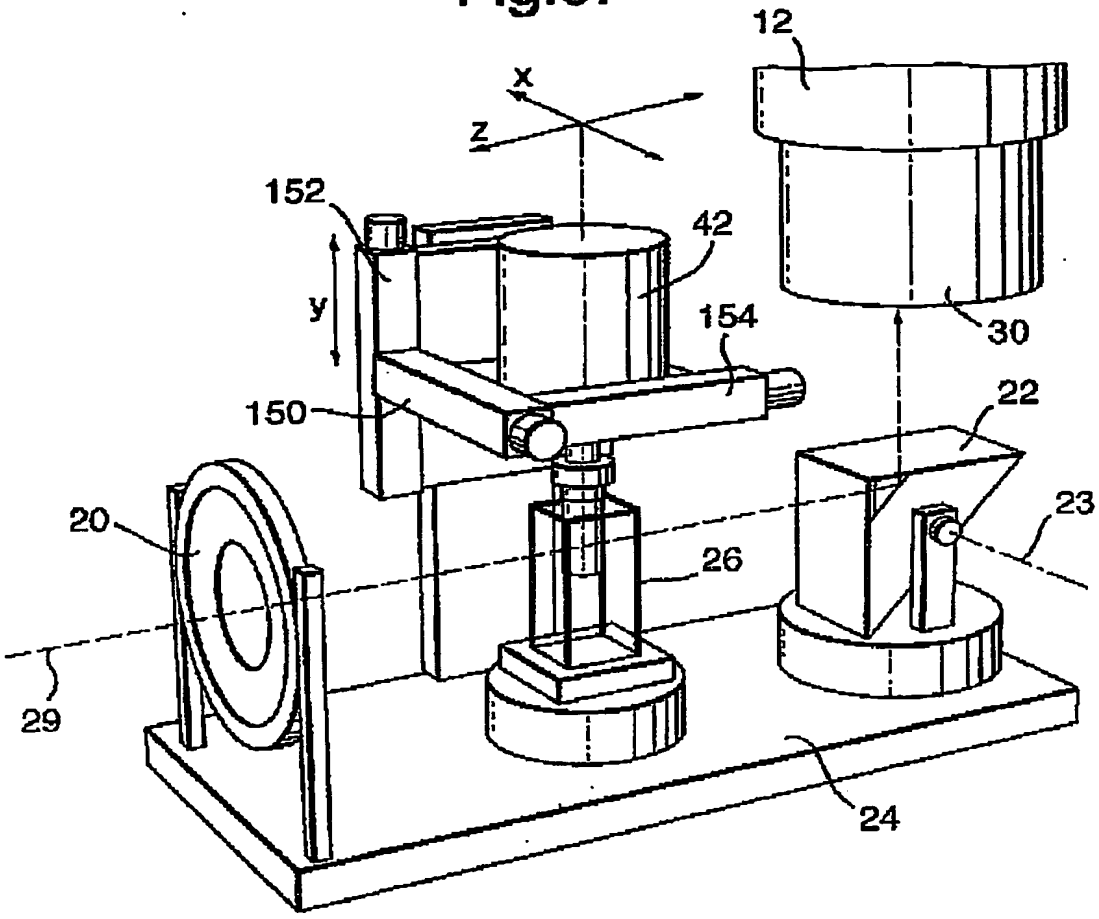


Fig.10.

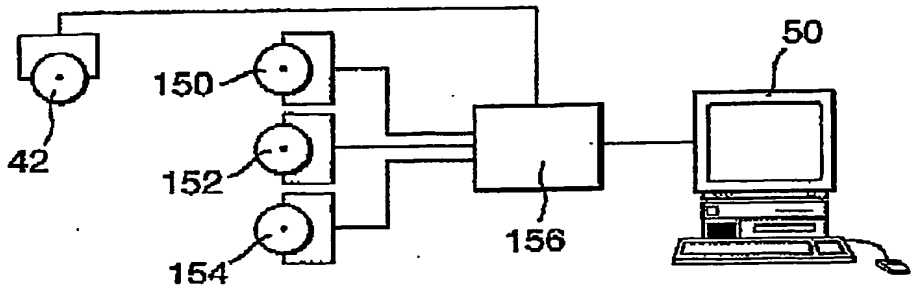


Fig.11(a)

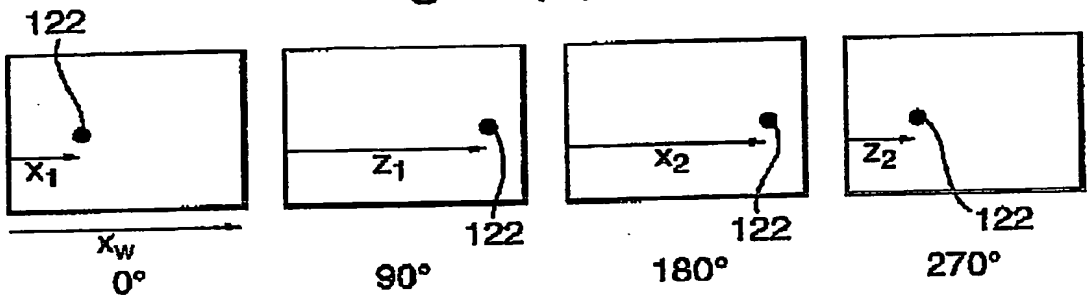


Fig.11(b)

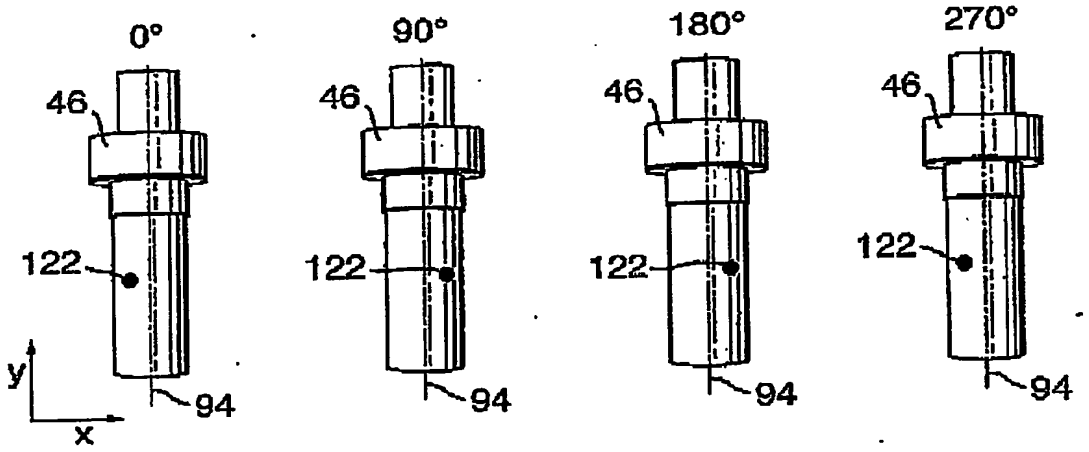


Fig.11(c)

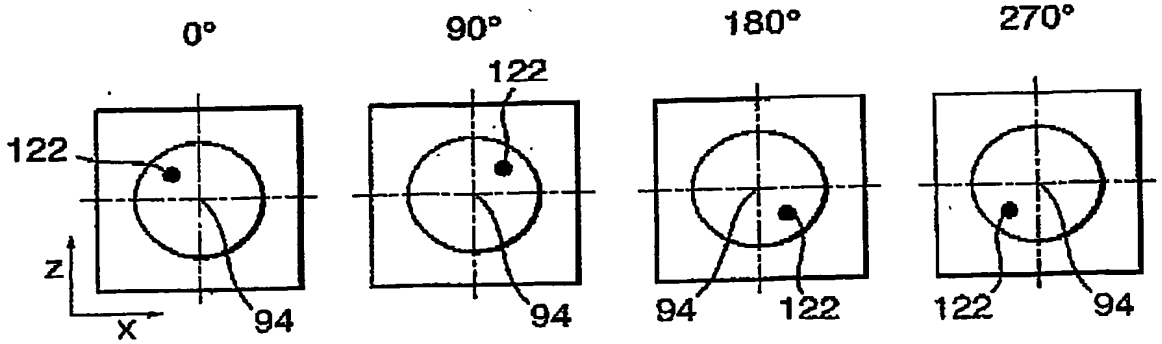


Fig.12(a)

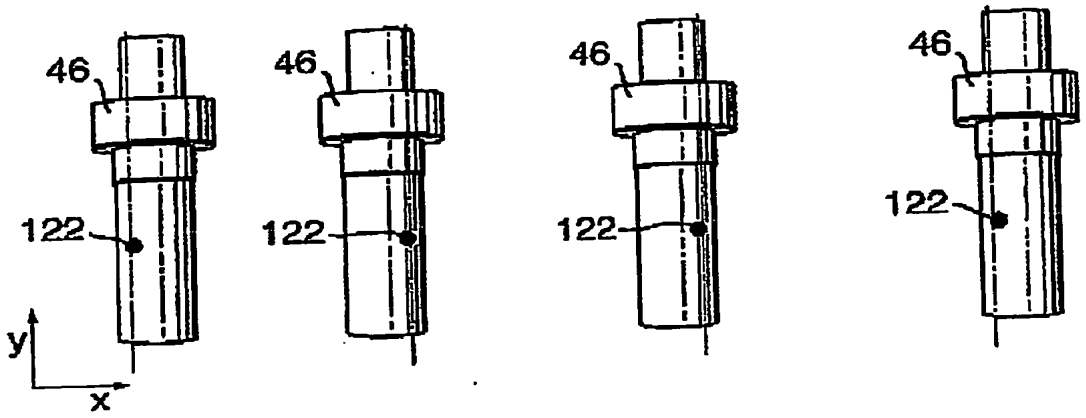


Fig.12(b)

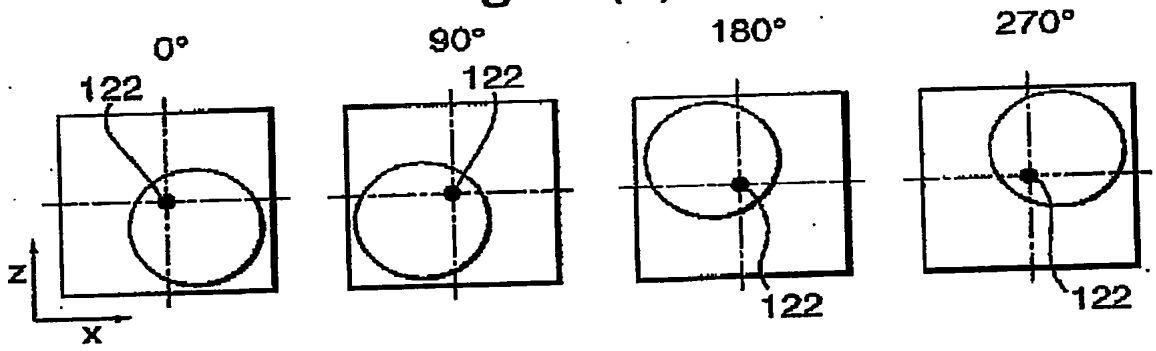


Fig.13(a)

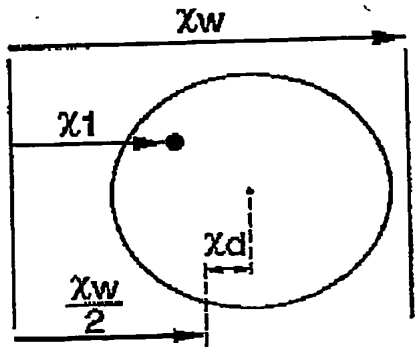


Fig.13(b)

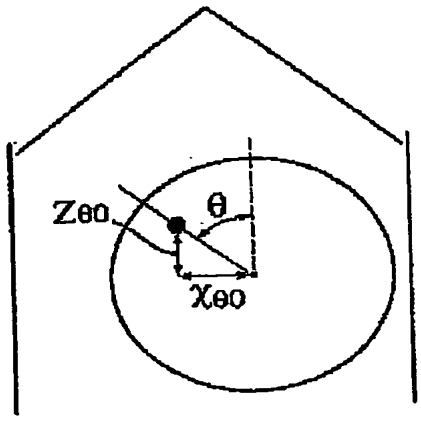


Fig.14.

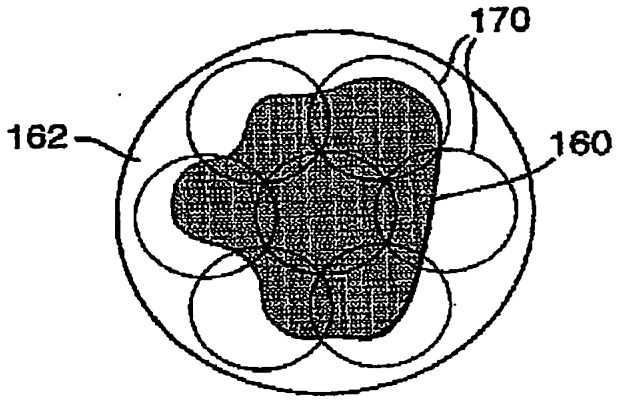


Fig.15.

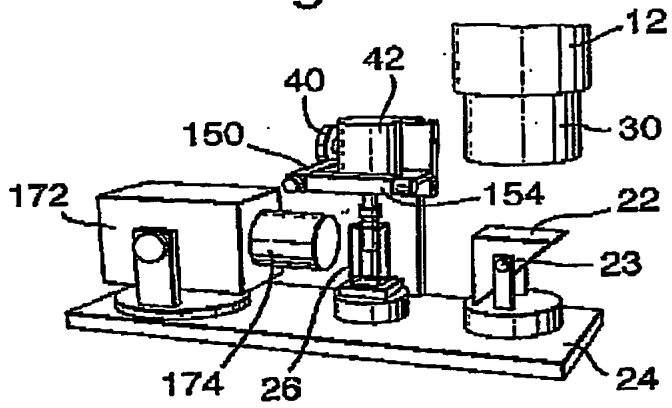
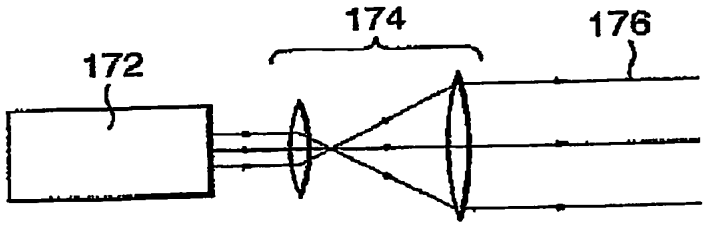
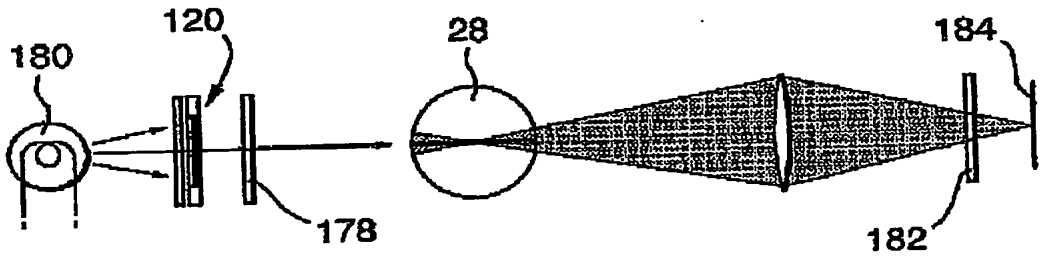


Fig.16.



10/45

Fig.17.



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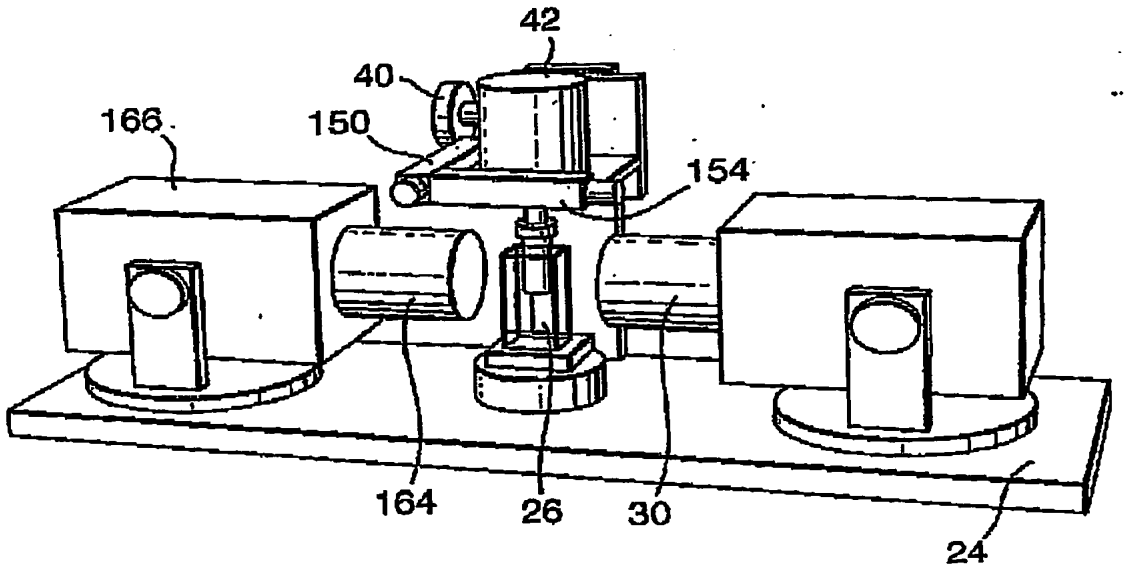


11/15

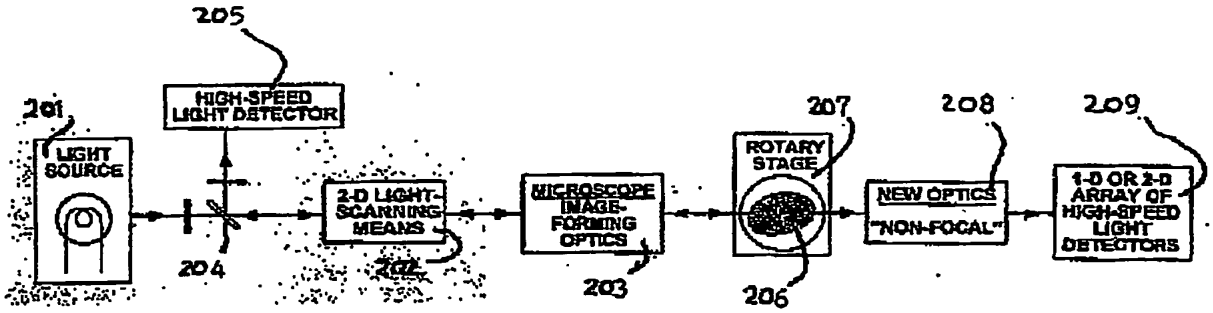


Figure 19

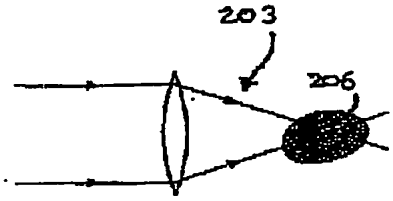


Figure 20a

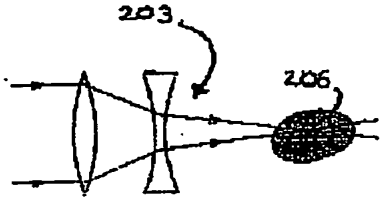


Figure 20b

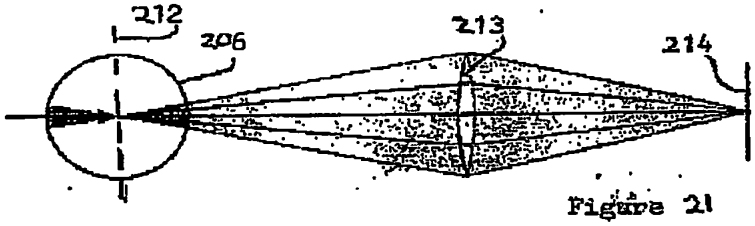


Figure 21

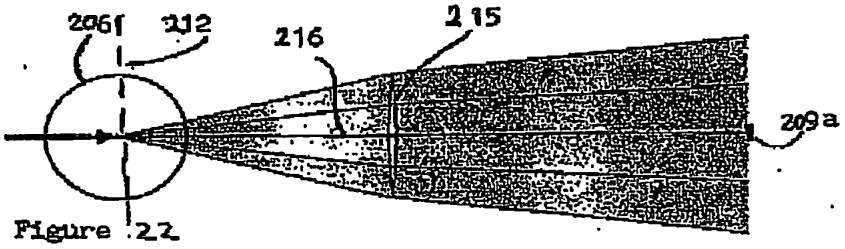
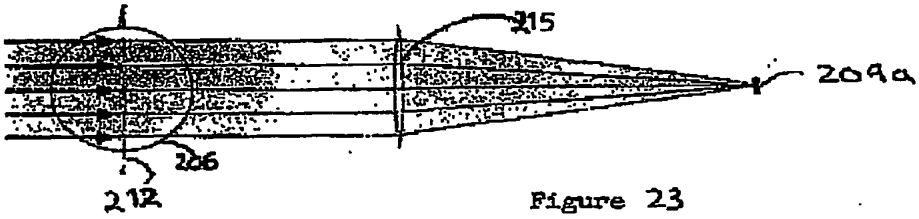


Figure 22



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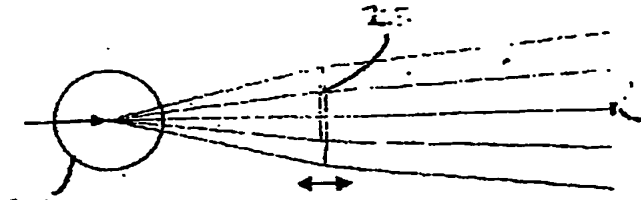

Figure 24a

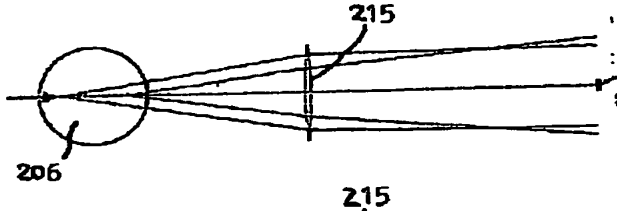

Figure 24b

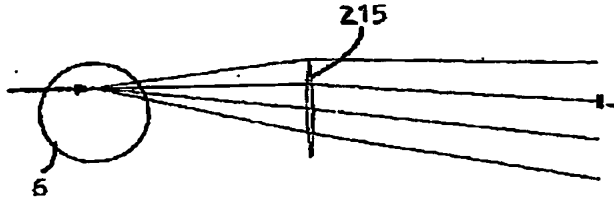

Figure 24c

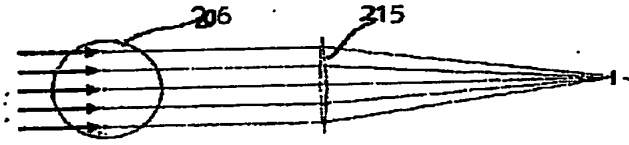

Figure 24d

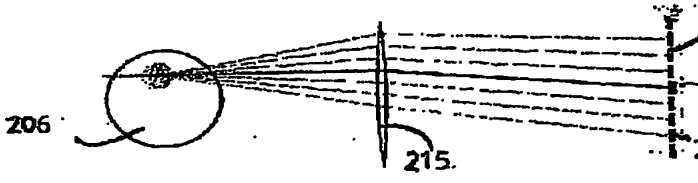

Figure 25a

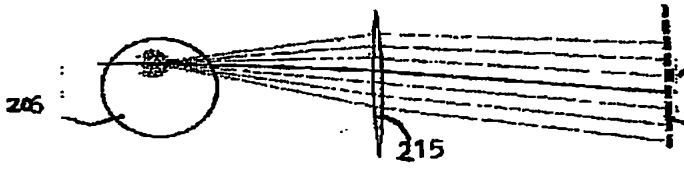

Figure 25b

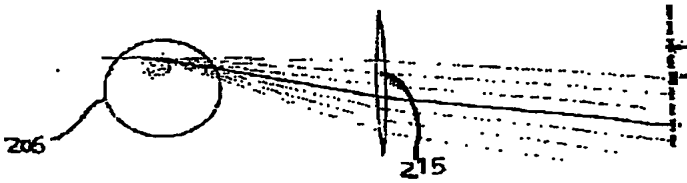

Figure 25c

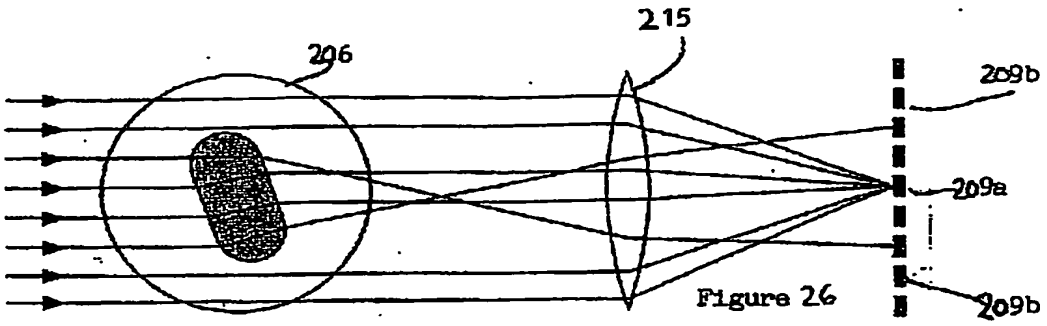

Figure 26

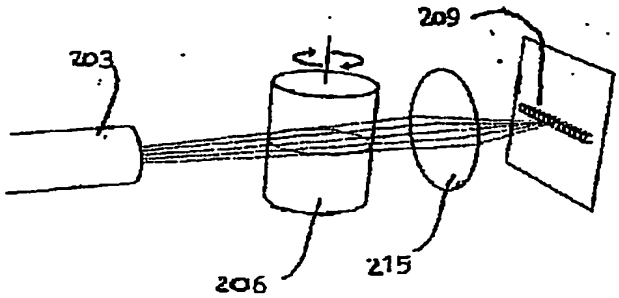

Figure 27

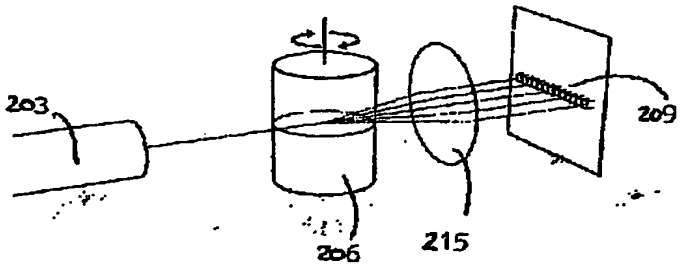

Figure 28

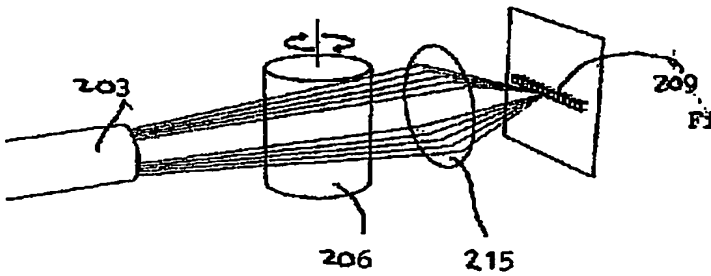

Figure 29

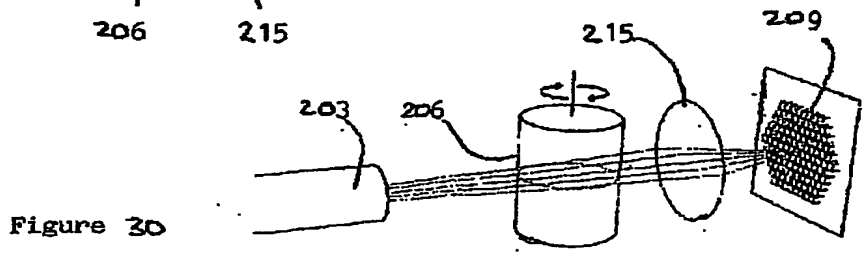

Figure 30

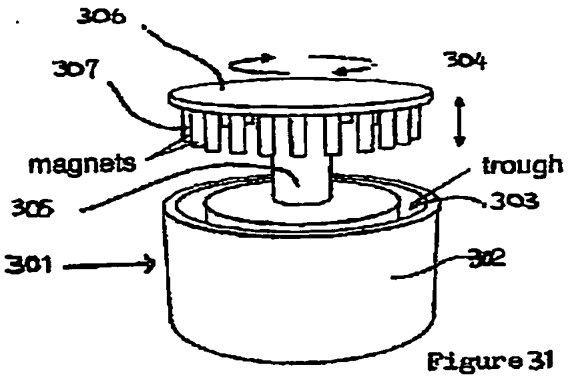

Figure 31

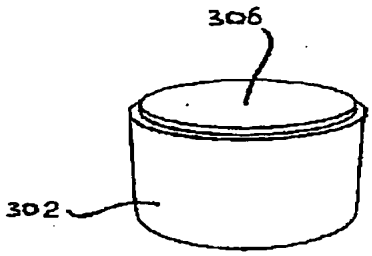

Figure 32

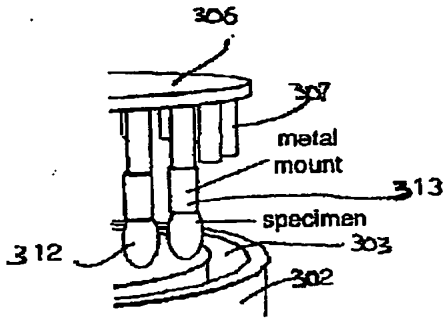

Figure 33

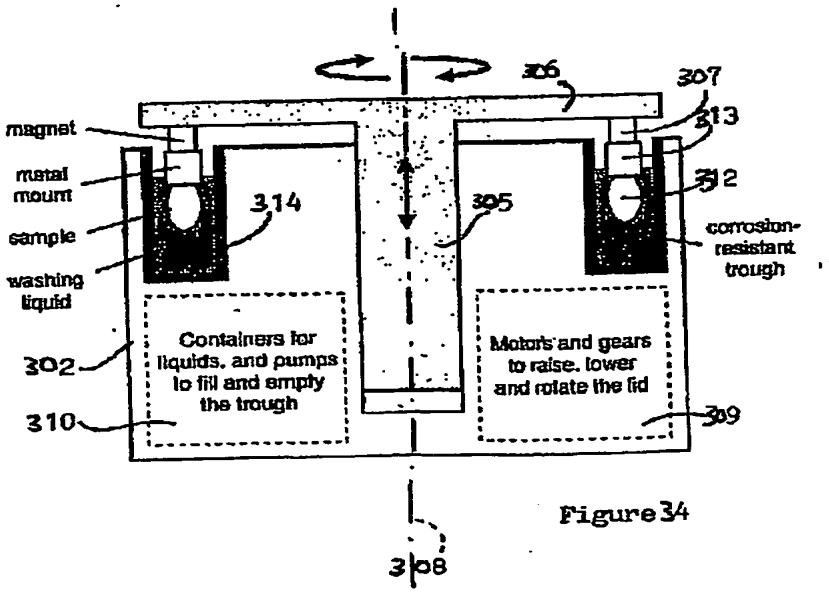

Figure 34

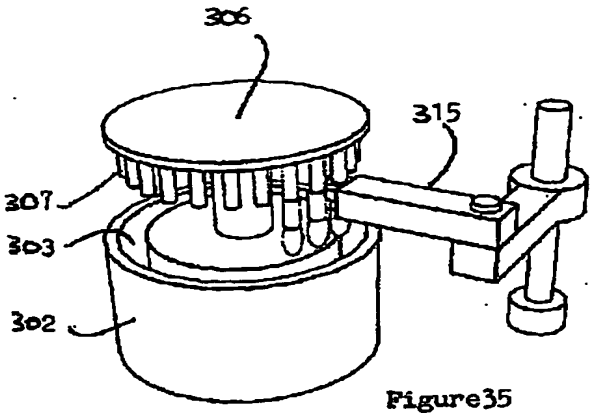

Figure35

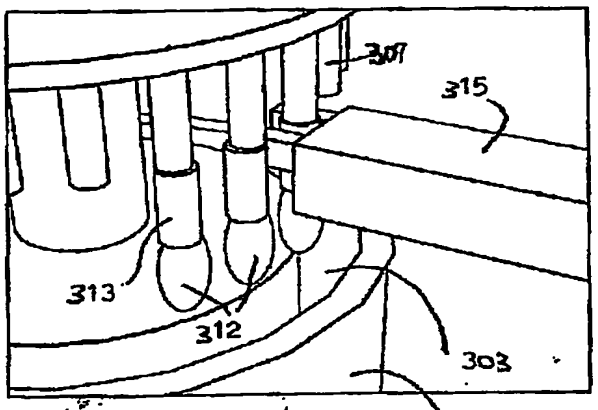

Figure36

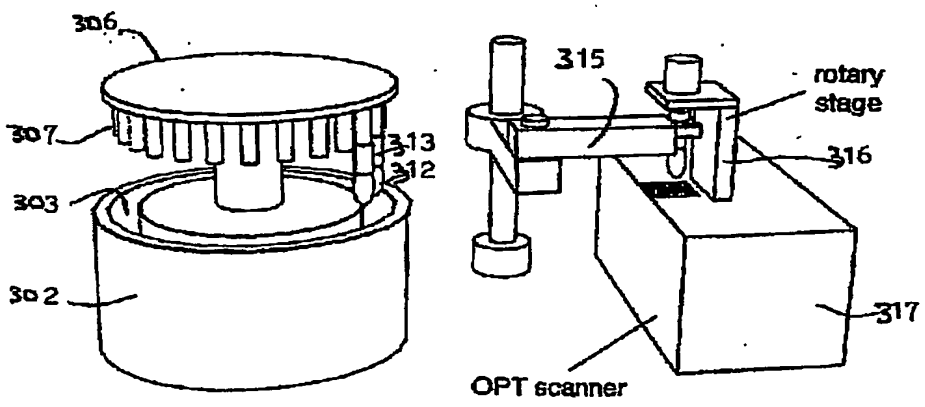

Figure37